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(54) **METHODS OF SCREENING AGENTS FOR ACTIVITY USING TELEOSTS**

SCREENINGVERFAHREN FÜR DIE AKTIVITÄT VON AGENZIEN UNTER VERWENDUNG VON TELEOSTEN

PROCEDES DE CRIBLAGE DESTINES A DETERMINER L'ACTIVITE D'AGENTS AU MOYEN DE POISSONS TELEOSTEENS

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Description

BACKGROUND OF THE INVENTION

[0001] Currently, searches for target-specific therapeutic and prophylactic compounds that have the ability to enhance or inhibit angiogenesis activity, enhance or inhibit cell death activity, and/or exhibit low toxicity comprise three major focuses of drug discovery and development. Angiogenesis plays an important role not only in the further development of the embryonic vasculature, but also in many post-natal processes, such as wound healing and tissue and organ regeneration. Angiogenesis has also been identified as a critical process for solid tumor growth. Furthermore, uncontrolled blood cell proliferation and excessive angiogenesis have been shown to constitute significant pathogenic components in numerous diseases, including rheumatoid arthritis, atherosclerosis, diabetes mellitus, retinopathies, psoriasis, and retrolental fibroplasia.

[0002] Methods of screening agents for an ability to inhibit or enhance angiogenesis activity would be useful in identifying those agents that would be effective in therapeutic or prophylactic treatment of a variety of diseases involving angiogenesis processes. For example, angiogenesis inhibition is a powerful potential approach for ameliorating cancer (Folkman, *New Eng. J. Med.* 333:1757-1763 (1995); Kerbel, *Nature* 390:355(1997)) and for reversing blood vessel loss associated with tissue ischemia, such as diabetic retinopathy (Bonn, *Lancet* 348:604 (1996); Breier *et al., Haemist.* 78(1):678-683 (1997). It appears that anti-angiogenic therapies do not induce acquired drug resistance (Boehm *et al., Nature* 390:404-407 (1997)) - a major problem with current cancer therapies. However, few therapeutic candidate molecules exist. It would therefore be desirable to provide methods of identifying compounds that inhibit angiogenesis and have therapeutic activities against diseases that would benefit from angiogenesis inhibition, such as cancer and diabetes. Similarly, methods of screening for compounds that enhance angiogenesis by stimulating blood vessel formation would be advantageous for use in minimally invasive approaches for improving circulatory function in various diseases, such as coronary artery disease, congestive heart failure, peripheral arterial disease, and peripheral venous disease. Unfortunately, many current assays for angiogenesis do not permit *in vivo* assessment of compounds or their side effects in whole animal models, or in multiple tissues or organs of animal models simultaneously and over time. In addition, many current assays for angiogenesis activity are not suitable for rapid, automated, or extensive compound screening, particularly screening of compound libraries containing numerous compounds, due to their complexity.

[0003] The search for compounds that can regulate promote or inhibit cell death has also been of vital interest. Necrosis and apoptosis are two known types of cell death. Necrosis involves the pathologic death of living tissue in a subject due to non-physiological injury to cells of the tissue. Apoptosis, which involves programmed cell death, is a physiological process that ensures that an equilibrium is maintained between cell proliferation and cell differentiation in most self-renewing tissues of multicellular organisms. Regulation of cell death activity (in particular, apoptosis) constitutes an important mechanism in therapeutic and prophylactic approaches to many diseases, including, *e.g.*, cancer and organ transplantation. Existing models for assessing apoptosis activity include the nematode worm, *C. elegans*. Although the nematode has many advantages as a model system, it is not the optimum model for evaluating vertebrate cell death activity or for use in screening compounds for potential therapeutic activity in vertebrates.

[0004] There are currently two approaches for detecting cell death activity in vertebrate hosts. The first approach uses standard cell culture techniques and typically relies on standard microplate readers to detect the death of cells cultured from an organism. A major drawback of the cell culture assay format is that it does not permit analysis of the effects of a compound on cell types that have not been cultured (*i.e.*, other cell types). It also does not allow evaluation of the effects of a compounds on specific tissues or organs or in an intact whole host *in vivo.* Furthermore, such an assay format does not permit the monitoring of cell death activities in multiple tissues, organs, or systems of a live host simultaneously or the continued monitoring of such activities over time. In addition, the cell culture assay approach does not allow for rapid or automated high-throughput screening of many compounds.

[0005] A second approach to detecting cell death activity utilizes a histochemical staining technique, designated terminal deoxyuridine nucleotide end labeling (TUNEL), to detect dead or dying cells in sectioned tissues of vertebrate embryos. Unfortunately, with this approach, only a single time point in the life cycle of the host can be examined; the death of cells in various tissues or organs of the subject over a period of time cannot be monitored. Because many degenerative diseases occur in stages, the ability to examine changes in the pattern of cell death activity caused by a compound and the duration of direct and side effects of the compound on multiple tissues and organs would represent a significant improvement over such methods. Moreover, because the TUNEL approach requires that cells be fixed for visualization, effects in a live animal cannot be monitored.

[0006] The identification of target-specific therapeutic and prophylactic compounds that exhibit low toxicity and/or side effects has also been focal point of drug discovery and development. Evaluation of the potential impact of different compounds on humans and animal health is a major component of risk assessment. There is increasing concern that current toxicity test procedures are inadequate. A number of cell-based *in vitro* toxicity screens have been developed. Significantly, however, these screens do not permit evaluation of the toxic effects of a compound *in vivo* on an intact

animal. Notably, these cell-based assays are designed at the molecular and cellular levels; as a result, determining the impact of a compound of interest on higher levels of cellular organization, such as the circulatory system and neurodevelopment, still requires subsequent whole animal testing. In addition, current screens do not permit the assessment of drug effects on all potential target cells, tissues, or organs of an animal. Nor can the effects of a compound on multiple target tissues and organs be studied simultaneously or over time using current assays. Underscoring the need for the development of more predictive and comprehensive toxicity screening methods, many compounds that pass preliminary cell-based testing fail final large animal toxicity testing, a prerequisite for eventual FDA approval. Furthermore, some potential therapeutic compounds that do not produce immediate lethality induce toxic effects in specific organs and tissues. There is a need for a cost-effective, comprehensive methods for screening compounds for toxic activity in whole animals and in one or more designated target tissues and organs *in vivo* and in cells *in vitro* and over time.

[0007] WO98/31787 describes assays for testing a compound's ability to affect cell death by contacting animal embryos, e.g. teleost embryos, with the compound and observing the resulting effects on apoptosis.

[0008] US 5,565,187 describes the use of a fluorescent dye complex and tracer (FITC-DX 150) to monitor effects of test compounds on capillary circulation and vascular permeability in teleosts, and its applicability to the study of physiological changes induced by the test compounds.

[0009] DE 4113393 describes a method for investigating angiogenesis-stimulating agents by providing epithelial cells within an inflammatory region of a tissue with suboptimal concentrations of growth factors and detecting their influence on epithelial cells.

[0010] US 5,712,395 describes an engineered subcutaneous xenograft model for investigating the regulation of angiogenesis.

SUMMARY OF THE INVENTION

[0011] The present invention relates generally to methods of screening an agent for an activity in a teleost. Methods of screening an agent for an angiogenesis activity *in vivo* or *in vitro* are provided as set out in the claims. Some such methods comprise administering the agent to a whole teleost *in vivo* and detecting a response in the teleost or in at least one tissue or organ of the teleost indicating the angiogenesis activity. Other such methods comprise administering the agent to cells of a teleost *in vitro* and detecting a response in such cells indicating the angiogenesis activity. In some such methods, the response is a reduction in blood vessel formation relative to an untreated teleost. In other such methods, the response is an increase in blood vessel formation relative to an untreated teleost.

[0012] In another aspect, the present invention provides methods of screening an agent for angiogenesis activity and toxicity *in vivo* or *in vitro*. Some such methods comprise administering the agent to a whole teleost *in vivo* and detecting a response in the teleost indicating angiogenesis activity and toxicity. Other such methods comprise administering the agent *in vitro* to cells of a teleost and detecting a response in the cells indicating angiogenesis activity and toxicity.

[0013] In yet another aspect, the present invention includes methods of screening an agent for angiogenesis activity and an effect on cell death activity *in vivo* or *in vitro*. Some such methods comprise administering the agent to a teleost *in vivo* and detecting a response in the teleost indicating angiogenesis activity and an effect on cell death activity. Other such methods comprise administering the agent *in vitro* to cells of a teleost and detecting a response in the cells indicating angiogenesis activity and an effect on cell death activity.

[0014] A further understanding of the nature and advantages of the inventions herein may be realized by reference to the detailed description of the specification and the associated figures.

BRIEF DESCRIPTION OF THE FIGURES

[0015]

Fig. 1 is a schematic diagram showing the processes of vasculargenesis and angiogenesis.

Figs. 2A, 2B, and 2C are photographs through a dissecting microscope showing lateral views of zebrafish embryos at 72 hours (hr) of development. The embryos have been stained with alkaline phosphatase (AP). Blood vessels are visualized by light microscopy after alkaline phosphatase staining. A control embryo (Fig. 2A) treated with 0.1% dimethyl sulfoxide (DMSO) has normal morphology and vessel formation. The subintestinal vessels (SIVs) (arrow) are in the characteristic pattern. An embryo treated with a fumagillin derivative at concentration of 10 micromolar (μM) (Fig. 2B) shows both developmental delay (reduced fin size and axial length) and loss of the SIVs (arrow). The pronepheric duct provides a positive control for AP staining (arrowhead). An embryo treated with a fumagillin derivative at a concentration of 100 μM (Fig. 2C) is dead. Fumagillin derivatives induce developmental delay and toxic response in the embryos. The eye (E), yolk (Y) and fin (F) of the embryos are labeled for orientation.

Scale bar = 100 μm.

Figs. 3A and 3B are photographs through a dissecting microscope showing two lateral views of zebrafish embryos at 72 hours of development. Each embryo has been stained with alkaline phosphatase. Fig. 3A represents a control; Fig. 3B shows a treated embryos treated with a compound from the NCI library. Both embryos are morphologically normal, however, the treated embryo has failed to form any SIVs (arrow) and shows a specific loss of the subintestinal vessels. The eye (E), yolk (Y) and fin (F) of the embryos are labeled for orientation. Scale bar = 100 μm.

Fig. 4 is a photograph through a dissecting microscope of a lateral view of an alkaline-phosphatase stained zebrafish embryo at 72 hours of development. The embryo has been treated with a compound which induced truncation, pericardial edema (arrow), and reduction of SIV formation (arrowhead). A loss of lateral vessels in the SIV basket is shown. The eye (E), yolk (Y) and fin (F) of the embryo are labeled for orientation. Scale bar = 100 μm.

Fig. 5 is a photograph through a dissecting microscope showing a lateral view of a zebrafish embryo treated with a compound which induced blebbing of the notocord (arrow), but did not effect SIV formation (arrowhead). The embryo been stained with AP. Axial defects do not usually effect angiogenesis. The eye (E), yolk (Y) and fin (F) of the embryo are labeled for orientation. Scale bar = 100 μm.

Figs. 6A-6D are photographs through a dissecting microscope showing lateral views of zebrafish embryos at 72 hours of development. Embryos have been stained with AP. When VEGF was injected into the yolk of an embryo (Figs. 6A and 6C), two angiogenic phenotypes were observed: 1) the appearance of long spikes projecting from the subintestinal vessel basket (long arrows); and 2) increased vessel diameters in the subintestinal basket (arrowheads). When VEGF was injected into the perivitelline space (Fig. 6D) of an embryo, we observed fusion of large vessels, inappropriate vessel formation (arrow), as well as heart (long arrow) and developmental defects. Control embryos (Fig. 6B), in which buffer was injected into either the yolk of perivitelline space, were normal. The eye (E), yolk (Y) and fin (F) of the embryos are labeled for orientation. Scale bar = 100 μm.

Fig. 7 is a photograph through a compound microscope (10x objective) of an alkaline phosphatase staining of zebrafish embryos at day three of development. These dorsal views of an untreated (top) and a treated (bottom) embryo show the effect of the anti-angiogenesis drug, Ovicillin, on the subintestinal veins (arrows). In addition to causing a reduction in the subintestinal vessels, the drug had other effects, including causing pericardial edema (arrowheads). In this figure, the letter "E" denotes the eye, and the letter "Y" denotes the yolkball.

Fig. 8 is a photograph through a compound microscope (20x objective) showing a wholemount RNA *in situ* hybridization with *flk-1* performed on a day one zebrafish embryo. In this lateral view of the trunk, intersomitic vessels (arrows), which are sprouting from the dorsal aorta (A), are labeled with the probe. Anterior is to the left and dorsal is up.

Fig. 9 is a microangiograph showing lateral profile of a zebrafish embryo at day three of development, depicting the normal vascular pattern, including the cranial (C), intersegmental (I) and subintestinal (S) vessels. The letter "H" denotes the heart, and the letter "E" denotes the eye. The data to construct the microangiograph was acquired from an epifluorescence microscope and processed using digital image processing software.

DEFINITIONS

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991). As used herein, the following terms and phrases have the meanings ascribed to them unless specified otherwise. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms and phrases are intended to have the following general meanings as they are used herein:

[0017] The term "subject" as used herein includes an animal. The term "animal" as used herein includes a vertebrate animal, such as a vertebrate fish. Vertebrate fish include teleosts, such as, *e.g.*, zebrafish, medaka, Giant rerio, and puffer fish.

[0018] The term "teleost" as used herein means of or belonging to the Telostei or Teleostomi, a group consisting of numerous fishes having bony skeletons and rayed fins. Teleosts include, for example, zebrafish, medaka, Giant rerio, and puffer fish.

[0019] The term "larva" or "larval" as used herein means the stage of any of various animals, including vertebrate animals, such as vertebrate fishes (including teleosts, such as, *e.g.*, zebrafish, medaka, Giant rerio, and puffer fish), between embryogenesis and adult.

[0020] "Angiogenesis activity" or "angiogenic activity" in reference to an agent is defined herein as the ability of the agent to enhance, inhibit, or prevent the formation or outgrowth of blood vessels or lymph vessels. Angiogenesis activity

or angiogenic activity in reference to a subject refers to activity associated with angiogenesis within a subject or organ (s) or tissue(s) of a subject or originating from within a subject or organ(s) or tissues(s) of the subject.

[0021] "Anti-angiogenesis activity" or "anti-angiogenic activity" in reference to an agent is defined herein as the ability of the agent to inhibit, prevent, or greatly reduce the formation or outgrowth of blood or lymph vessels, or destroy such vessels during sprouting or outgrowth. Anti-angiogenesis activity or anti-angiogenic activity in reference to a subject refers to activity associated with anti-angiogenesis within a subject or organ(s) or tissue(s) of a subject or originating from within a subject or organ(s) or tissues(s) of the subject.

[0022] The term "apoptotic activity" or "apoptosis activity" in reference to an agent is defined herein as the ability of the agent to enhance, inhibit, or prevent apoptosis. Apoptotic activity or apoptosis activity in reference to a subject refers to activity associated with the death of cells within a subject or organ(s) or tissue(s) of a subject or originating from within a subject or organ(s) or tissues(s) of the subject.

[0023] "Cell death activity" in reference to an agent is defined herein as the ability of the agent to enhance, inhibit, or prevent the death of one or more cells within a subject or organ(s) or tissue(s) of a subject or originating from within a subject or organ(s) or tissues(s) of the subject. Cell death activity in reference to a subject refers to activity associated with the death of cells within a subject or organ(s) or tissue(s) of a subject or originating from within a subject or organ (s) or tissues(s) of the subject.

[0024] The term "necrotic activity" or "necrosis activity" in reference to an agent is defined herein as the ability of the agent to enhance, inhibit, or prevent necrosis.

[0025] An "effect on cell death activity" as used herein refers to the way in which an agent acts upon or influences cell death activity in a subject. Such effects include an ability to enhance or inhibit cell death activity in the subject, as indicated or manifested by, for example, a clinical manifestation, characteristic, symptom, or event that occurs or is observed in, associated with, or peculiar to death of cells in a subject.

[0026] An "effect on apoptotic activity" as used herein refers to the way in which an agent acts upon or influences apoptotic activity in a subject. Such effects include an ability to enhance or inhibit apoptotic activity in the subject, as indicated or manifested by, for example, a clinical manifestation, characteristic, symptom, or event that occurs or is observed in, associated with, or peculiar to apoptosis of cells in a subject.

[0027] An "endogenously occurring" as used herein means occurring originating from within.

[0028] The term "gene" is used broadly to refer to any segment of DNA associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include non-expressed DNA segments that, for example, form recognition sequences for other proteins. The term "nucleic acid" or "nucleic acid segment" refers to a deoxyribonucleotide or ribonucleotide and polymer thereof which is in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues (synthetic and naturally occurring) of nucleotides, which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to the reference nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0029] The term "isolated nucleic acid" or "isolated nucleic acid segment" means a single- or double-stranded nucleic acid (*e.g.,* an RNA, DNA, or a mixed polymer), which is substantially separated from other genome DNA sequences as well as proteins or complexes such as ribosomes and polymerases, which naturally accompany a native sequence. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule. An "isolated polypeptide" or protein carries a similar meaning with the polypeptide or protein being substantially separated from any cellular contaminants and components naturally associated with the protein *in vivo*.

[0030] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

[0031] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0032] A "chimeric molecule" as used herein refers to a linked molecule obtained after conjugation of two or more different types of molecules (e.g., lipids, glycolipids, peptides, proteins, glycoproteins, carbohydrates, nucleic acids, natural products, synthetic compounds, organic molecule, inorganic molecule, *etc.*).

[0033] The term "normal blood vessel formation" as used herein refers to the typical, usual, or natural process of forming or producing blood vessels in a subject.

[0034] The term "gene expression profile" or "gene expression pattern" as used herein means a profile or pattern based on the detection of mRNA for each gene to be included in the profile or pattern. mRNA can be detected at a particular time or under a particular condition(s). mRNA is extracted from cells, tissues, organs, or an entire organism of interest and detected. The amount or level of mRNA for a particular gene can be determined quantitatively.

[0035] The term "protein expression profile" or "protein expression pattern" as used herein means a profile or pattern based on the detection of a protein. The protein can be detected at a particular time or under a particular condition(s). Protein is extracted from cells, tissues, organs, or an entire organism of interest and detected. The amount or level of protein can be determined quantitatively.

[0036] The term "agent" includes any element, compound, or entity, including, but not limited to, *e.g.*, pharmaceutical, therapeutic, pharmacologic, environmental or agricultural pollutant or compound, aquatic pollutant, cosmeceutical, drug, toxin, natural product, synthetic compound, or chemical compound.

[0037] The term "natural compound" as used herein includes a molecule isolated, extracted, or purified from a plant, animal, yeast, bacterium, or other microorganism. A natural compound includes, *e.g.*, among other things, organic molecules belonging to the broad biochemical classes of peptides, proteins, glycoproteins, nucleic acids, carbohydrates, lipids, fats, glycolipids, as well as more complex molecules which comprise, *e.g.*, elements of more than one of these basic biochemical classes.

[0038] The term "synthetic compound" as used herein includes a molecule synthesized *de novo* or produced by modifying or derivatizing a natural compound.

[0039] "Developmental defect" as used herein means a deficiency, imperfection, or difference in the development of a tissue, organ, or other bodily component of an animal relative to normal development. Such a defect is identified as a change, difference, or lack of something necessary or desirable for completion or proper operation in the development of a tissue, organ, or other bodily component of the animal as compared with normal development of the component. Developmental defects include, for example, the failure of organ to develop properly, excess or reduced cell proliferation as compared to normal cell proliferation, and the malfunctioning of an organ or tissue.

[0040] Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry described below are those well known and commonly employed in the art. Standard techniques such as described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2nd ed. 1989) and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vols. 1-3 (Virginia Benson Chanda ed., John Wiley & Sons, 1994-1998), each of which is incorporated herein by reference in its entirety for all purposes, are used for recombinant nucleic acid methods, nucleic acid synthesis, cell culture, and transgene incorporation, *e.g.*, electroporation, injection, ingestion, and lipofection. Electroporation techniques utilize a pulse of high electrical current to introduce molecules of interest into cells, tissues, or organisms. Lipofection employs lipid-like cationic molecules that interact strongly with cell membranes, destabilizing them locally, thereby allowing DNA and RNA entry into cells. Generally, oligonucleotide synthesis and purification steps are performed according to the specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

[0041] The term "transgenic" in reference to an organism or animal includes those organisms or animals that have developed from a fertilized egg, into a chromosome of which a foreign gene has been inserted. Such transgenic organisms and animals carry the foreign gene insert in every cell. Transgenic organisms and animals are created by using known techniques (*see, e.g.*, Sambrook, *supra* and BIOCHEMISTRY WITH CLINICAL CORRELATIONS (T. Devlin ed., 3d ed. 1992), which is incorporated herein by reference in its entirety for all purposes). Transgenic organisms and animals can be used to study different aspects of the foreign gene, including the analysis of DNA regulatory elements, expression of proteins during differentiation, tissue specificity, and the potential role of oncogene products on growth, differentiation, and the induction of tumorigenesis. A "transgene" is a gene, in original or modified form, that has been introduced into an organism or animal that does not naturally have such gene. A "mosaically expressing transgene" is a transgene that is expressed randomly in a subset of the cells of the transgenic organism or animal. An "exogenous gene" is a gene from an organism or animal that does not belong to the species into which the gene has been introduced. A "transient transgenic animal" is transgenic animal which carries an introduced gene that is not inserted into a chromosome.

[0042] The term "founder fish" as used herein refers to the fish from which a line of fish is generated. Usually, a founder fish is an individual fish which carries a unique mutation and which is used to generate progeny that also carry the mutation.

[0043] A "physiological activity" in reference to an organism is defined herein as any normal processes, functions, or activities of a living organism.

[0044] A "prophylactic activity" is an activity of, for example, an agent, gene, nucleic acid segment, pharmaceutical,

substance, compound, or composition which, when administered to a subject who does not exhibit signs or symptoms of a disease or exhibits only early signs or symptoms of a disease, diminishes, decreases, or prevents the risk in the subject of developing pathology.

[0045] A "therapeutic activity" is defined herein as any activity of *e.g.*, an agent, gene, nucleic acid segment, pharmaceutical, therapeutic, substance, compound, or composition, which diminishes or eliminates pathological signs or symptoms when administered to a subject exhibiting the pathology. The term "therapeutically useful" in reference to an agent means that the agent is useful in diminishing, decreasing, treating, or eliminating pathological signs or symptoms of a pathology or disease.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

I. GENERAL

[0046] The present invention is directed to methods of screening an agent for an activity. In Section II of the application, methods of screening an agent for an ability or capacity to enhance, inhibit, or block angiogenesis activity are discussed.

A. Animal Models

[0047] The methods of the present invention, which are directed to screening agents for activities (*e.g.*, angiogenesis activity), are generally applicable for use in a various animals, including vertebrate animals, such as fish. Various species of fish are suitable, including teleosts. Suitable teleosts include, for example, zebrafish (*Danio rerio*), Medaka, Giant rerio, and puffer fish. Typically, animal models of the present invention are fish that are transparent or translucent (*i.e.*, optically clear) in at least one of the following stages: the embryonic, larval, or adult stage.

[0048] Certain teleosts, including the zebrafish, Medaka, Giant rerio, and puffer fish, offer important advantages over other animal model systems for use in screening methods of the present invention. First, these teleosts are vertebrates whose genetic makeup is more closely related to that of man than are other models, such as the *Drosophila* and nematode. All essential components of human form and organ development are mimicked in these teleosts and the morphological and molecular bases of tissue and organ development are either identical or similar to other vertebrates, including man. Chen and Fishman, *Development* 123:293-302 (1996); Granato and Nusselien-Volhard, *Cur. Op. Gen. Dev.* 6:461-468 (Wylie ed. 1996). As a result, these teleosts serve as an excellent model for the study of vertebrate development and human disease states.

[0049] Second, these teleosts provide advantageous animal models because their embryos are very transparent. Given the transparency of the embryo, angiogenesis activity, cell death activity (*e.g.*, apoptosis and necrosis), and toxic activity produced by administered agents can be detected and diagnosed much more rapidly than in non-transparent animals. These activities can also be detected in the more mature larval and adult forms of the zebrafish, though somewhat less readily as such forms become progressively less optically clear. These activities can also be detected *in vivo* in all three forms or in cells thereof *in vitro.* By contrast, the mouse, which is commonly used as an animal model system, is an opaque animal and does not allow a similar rapid or *in vivo* assessment of phenotypic or developmental changes, including those associated with cell death, angiogenesis, or toxicity, in whole animal or whole organs or tissues. Significantly, precursor tissues and components of the brain, eyes, heart, and musculature of these teleosts are detected and visualized much more easily and quickly in the transparent teleosts than in other systems, including other vertebrate systems (such as the mouse) by a variety of detection techniques, including, *e.g.*, light microscopy, fluorescence microscopy, colorimetry, chemiluminescence, digital imaging, microplate reader techniques, *in situ* hybridization of RNA, *etc.*

[0050] Another important advantage of teleosts over other animal models is that teleosts develop much more rapidly than do other animal models. In general, the body plan, organs, tissues, and other systems of teleosts develop much more rapidly than do such components in other vertebrate model systems (*e.g.*, the mouse). The entire vertebrate body plan of the zebrafish, for example, is typically established within 24 hours. A functioning cardiovascular system is evident in the zebrafish within the first 24 hours of development. Stainier and Fishman, *Trends Cardiovasc. Med.* 4: 207-212 (1994). The remaining organs of the zebrafish, including the gut, liver, kidney, and vasculature, are established within 48 hours. The hatched zebrafish embryo nearly completes morphogenesis within 120 hours, thereby making it highly accessible to manipulation and observation and amenable to high-throughput automated observation and detection procedures.

[0051] The cell death activity, angiogenesis activity, and toxic activity of an agent and responses indicating these activities can be monitored in whole teleosts and/or *in vivo* or in cells thereof *in vitro* over time - a procedure not possible or readily practiced with other animal embryos which develop *in utero,* such as the mouse. Moreover, the effects of an agent on the whole teleost embryo or on more than one system (*e.g.*, cardiovascular system, enteric system, and

musculature system), organ, or tissue can be detected simultaneously using transparent teleosts. The persistence of such effects can be monitored by using simple visualization methods and over selected time intervals. By comparison, it is extremely difficult to detect and assess developmental and phenotypic changes in organs, tissues, and systems (such as inhibition or enhancement of angiogenesis, cell death or toxic activity due to an agent) over time in animals which develop *in utero.* Mouse embryos, for example, must be removed from the mother - a labor intensive procedure - before an assay can be performed.

[0052] Teleosts also offer the advantage that agents to be evaluated for toxic effects can be administered directly to the developing teleost. Direct introduction of candidate compounds is hindered in animals which develop *in utero,* such as the mouse embryo. Further, the teleost embryo is an intact, self-sustaining organism. It is different from a mouse embryo, for example, which because it is physically removed from its mother's womb, it is not self-sustaining or intact; a mouse embryo would function more as an "organ" culture or the like.

[0053] Another significant advantage is cost. Mouse assays are expensive, primarily due to the cost of breeding and maintenance and the need to manually perform injections and subsequent analysis. The average cost of a commercial mouse tumor assay is approximately $2,900 ($1,600 per government). In contrast, teleosts, such as zebrafish, are comparatively inexpensive to generate and maintain. For example, the estimate cost of a zebrafish assays is less than $100. A single mating of a zebrafish produces 100-200 eggs. Inbred strains are available and thousands of zebrafish can be raised inexpensively in a small room of aquaria. Moreover, teleost eggs, including those of the zebrafish, are externally fertilized. Teleost embryos (such as zebrafish) can survive by diffusion of oxygen from the water and nutrients from the yolk and thus even the absence of the entire circulatory system is well tolerated during early development. Weinstein *et al.*, *Nature Med.* 1:1143-1147 (1995).

[0054] Additionally, single whole teleost embryos can be maintained *in vivo* in fluid volumes as small as 100 microliters for the first six days of development. Intact live embryos can be kept in culture in individual microtiter wells or multi-well plates. Test compounds can be added directly to the solution in which the fish is immersed. Compounds permeate the intact embryo directly, making this multi-well format particularly attractive for high through-put and automated compound screening. Both the therapeutic activities and side effects (*e.g.*, toxicity) of a drug can be assayed in the fish simultaneously *in vivo*.

[0055] The teleosts used with the screening methods of the invention are typically early-stage teleost embryos; however, transparent larval or adult teleosts can also be used. Wildtype strains of teleosts are usually employed. Wildtype strains are typically maintained for about one year, after which time fertility decreases. Mutant strains of teleosts (such as zebrafish) can be used to assess, *e.g.*, the interaction between therapeutic agents and specific genetic deficiencies. The teleost can contain a mutation in a selected gene. The mutation can be a heritable mutation, including, *e.g.*, a heritable mutation associated with a developmental defect. The teleost can also be transgenic.

B. Agents to be Screened

[0056] A variety of agents from various sources can be screened for enhancing or inhibiting angiogenesis activity by using the methods of the present invention. Agents to be screened can be naturally occurring or synthetic molecules. Agents to be screened can also obtained from natural sources, such as, *e.g.*, marine microorganisms, algae, plants, fungi, *etc.* Alternatively, agent to be screened can be from combinatorial libraries of agents, including peptides or small molecules, or from existing repertories of chemical compounds synthesized in industry, *e.g.*, by the chemical, pharmaceutical, environmental, agricultural, marine, cosmeceutical, drug, and biotechnological industries. Agents can include, *e.g.*, pharmaceuticals, therapeutics, environmental, agricultural, or industrial agents, pollutants, cosmeceuticals, drugs, organic compounds, lipids, glucocorticoids, antibiotics, peptides, proteins, sugars, carbohydrates, chimeric molecules, *etc.*

[0057] Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, proteins, nucleic acids, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, behzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax WO 93/06121, Columbia University, WO 94/08051, Pharmacopeia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated herein by reference in its entirety for all purposes). Peptide libraries can also be generated by phage display methods. *See, e.g.*, Devlin, WO 91/18980. Compounds to be screened can also be obtained from governmental or private sources, including, *e.g.*, the National Cancer Institute's (NCI) Natural Product Repository, Bethesda, MD, the NCI Open Synthetic Compound Collection, Bethesda, MD, NCI's Developmental Therapeutics Program, or the like.

C. Administration of Agents

[0058] Agents to be screened for an effect on angiogenesis activity, can be administered to the teleost by adding

the agent directly to the media containing the live teleost. Alternatively, the agent can first be dissolved in the media and the live teleost submerged in the media subsequently. Such approaches have been used to introduce anesthetics and other chemicals to fish embryos. *See, e.g.*, M. Westerfield, THE ZEBRAFISH BOOK: A GUIDE FOR THE LABORATORY USE OF ZEBRAFISH (3d. ed. 1995), which is incorporated herein in its entirety for all purposes. Agents can also be administered to the teleost by using microinjection techniques in which the agent is injected directly into the live teleost. For example, agents can be injected into either the yolk or body of a teleost embryo or both.

[0059] Agents can also be administered to teleosts by electroporation, lipofection, or ingestion or by using biolistic cell loading technology in which particles coated with the biological molecule are "biolistically" shot into the cell or tissue of interest using a high-pressure gun. Such techniques are well known to those of ordinary skill in the art. *See, e.g.*, Sambrook *et al.*, *supra*; Chow *et al.*, *Amer. J. Pathol.* 2(6):1667-1679 (1998).

[0060] Agents can be administered alone, in conjunction with a variety of solvents (*e.g.,* dimethylsulfoxide or the like) or carriers (including, *e.g.*, peptide, lipid or solvent carriers), or in conjunction with other compounds.

[0061] Agents can be administered to the teleost before, at the same time as, or after administration of a dye used for detection of the response in the animal indicating a specific activity (*e.g.*, cell death activity, angiogenesis activity, toxic activity).

D. Administration of Dyes

[0062] A dye used in methods of screening agents for an activity (*e.g.*, cell death activity, angiogenesis activity, toxic activity) can be administered to the teleost by adding the agent directly to the media containing the live teleost. Alternatively, the dye can first be dissolved in the media and the live teleost submerged in the media subsequently. *See, e.g.*, Westerfield, *supra*. Dyes can also be administered to the teleost by using microinjection techniques in which the dye is injected directly into the live teleost. Dyes can be injected into either the yolk or body of a teleost embryo or both.

[0063] Dyes can be administered alone, in conjunction with a variety of solvents (*e.g.,* dimethylsulfoxide or the like), or in conjunction with other dyes. Dyes can be administered to the teleost before, at the same time as, or after administration of a dye used for detection of the response in the teleost indicating a specific activity (*e.g.*, cell death activity, angiogenesis activity, toxic activity). When fluorescent dyes are used (*e.g.*, unsymmetrical cyanine dye, such as a quinolium dye) for detection of an activity (*e.g.,* cell death activity), the dye is preferably administered prior to administration of the agent.

E. Detecting Agent Activity and Responses in Teleosts

[0064] A variety of techniques can be used together or separately to generate a signal and to detect and assess the effect of an agent on cell death activity or angiogenesis activity or toxic activity of an agent. Signals can be generated by, for example, *in situ* hybridization, antibody staining of specific proteins (*e.g.*, antibody markers that label signaling proteins involved in angiogenic vessel formation in teleost, including VEGF and Ang1 and 2; terminal deoxyuridine nucleotide end labeling to detect dead or dying cells, *etc.*). Responses indicating toxic or angiogenic activity or an effect of cell death activity can be detected by, *e.g.,* visual inspection, colorimetry, fluorescence microscopy, light microscopy, chemiluminescence, digital image analyzing, standard microplate reader techniques, fluorometry, including time-resolved fluorometry, visual inspection, CCD cameras, video cameras, photographic film, or the use of current instrumentation such as laser scanning devices, fluorometers, photodiodes, quantum counters, plate readers, epifluorescence microscopes, scanning microscopes, confocal microscopes, flow cytometers, capillary electrophoresis detectors, or by means for amplifying the signal such as a photomultiplier tube, *etc.* Responses can be discriminated and/or analyzed by using pattern recognition software. Agents are identified and selected using the screening methods according to the activities and responses they produce.

[0065] Changes in the distribution of a protein both spatially and temporally, including a complete absence of a protein, can be detected and protein expression profiles can be generated. Changes in a level of an enzyme or enzymatic activity within the intact teleost can also be detected by various means, including, *e.g.*, alkaline phosphatase staining and use of streptavidin (avidin) conjugated reporter enzyme to detect naturally biotinylated carboxylase enzymes in the liver, gut, and digestive tube of animals.

F. Automated Methods

[0066] In addition to manual screening methods, the present invention also provides methods for rapid screening of agents for activities, such as angiogenesis activity, using automated procedures. Such automated methods can be readily performed by using commercially available automated instrumentation and software and known automated observation and detection procedures. Multi-well formats are particularly attractive for high through-put and automated compound screening. Screening methods can be performed, for example, using a standard microplate well format,

with a whole zebrafish embryo in each well of the microplate. This format permits screening assays to be automated using standard microplate procedures and microplate readers to detect enhancement or inhibition of angiogenesis activity in the zebrafish embryos in the wells. A microplate reader includes any device that is able to read a signal from a microplate (*e.g.*, 96-well plate), including fluorometry (standard or time-resolved), luminometry, or photometry in either endpoint or kinetic assays. Using such techniques, the effect of a specific agent on a large number of teleosts (*e.g.*, teleost embryos) *in vivo* or *in vitro* can be ascertained rapidly. In addition, with such an arrangement, a wide variety of agents can be rapidly and efficiently screened for their respective effects on the cells of teleosts contained in the wells.

[0067] Sample handling and detection procedures can be automated using commercially available instrumentation and software systems for rapid reproducible application of dyes and agents, fluid changing, and automated screening of target compounds. To increase the throughput of a compound administration, currently available robotic systems (e.g., the BioRobot 9600 from Qiagen, the Zymate from Zymark or the Biomek from Beckman Instruments) - most of which use the multi-well culture plate format - can be used. The processing procedure involves a large number of fluid changes that must be performed at defined timepoints. Non-automated throughput is typically 5 microtiter plates per investigator (400 teleost embryos and 20 compounds) per week based on using a 96-well plate with 1 embryo per well and screening 2 concentrations with 10 embryos per concentration. Using currently available fluid handling hardware (*e.g.*, Bodhan Automation, Inc., Zymark) and our standard sample handling procedures, 50-100 plates per day (4800-9600 teleost embryos and 200-400 compounds) can be processed. Incorporation of commercially available fluid handling instrumentation significantly reduces the time frame of manual screening procedures and permits efficient analysis of many agents, including libraries of agents.

II. METHODS OF SCREENING AN AGENT FOR AN EFFECT ON ANGIOGENESIS ACTIVITY

A. Angiogenesis

[0068] The formation and establishment of a vascular supply is an essential requirement for the cellular inflow of nutrients, outflow of waste products, and gas exchange in most tissues and organs. Two processes for such blood vessel development and differentiation have been identified. One process of vascularization, termed "vasculogenesis," occurs in the embryo and consists of the *in situ* differentiation of mesenchymal cells into hemoangioblasts. Hemoangioblasts are the precursors of both endothelial cells and blood cells. The second process, termed "angiogenesis," involves the formation of new blood and lymph vessels from preexisting endothelium. In this process, tissues and organs are vascularized by sprouting in which smaller vessels extend from larger vessels and penetrate a specific tissue. Fouquet *et al.*, *supra*. Angiogenesis also involves the migration and proliferation of endothelial cells, their differentiation into a tube-like structure, and the production of a basement membrane matrix around the vessel. Herbert *et al.*, *L. Cell. Biol.* 106:1365-1373 (1988).

[0069] Methods for screening agents for inhibition or enhancement of angiogenesis activity are useful in identifying agents that would be effective in therapeutic or prophylactic treatment of a variety of diseases involving angiogenic processes.

B. Blood Vessel Formation

[0070] New blood vessels form during normal tissue growth and repair in a series of sequential steps: an endothelial cell which forms the wall of an existing small blood vessel (capillary) becomes activated, secretes enzymes that degrade the extracellular matrix (the surrounding) tissue, invades the matrix, and begins dividing. Eventually, strings of new endothelial cells organize into hollow tubes, creating new networks of blood vessels that make tissue and repair possible. Ordinarily, endothelial cells lie dormant. However, when necessary, short bursts of blood vessel growth occur in localized parts of tissues. New capillary growth is tightly controlled by a finely tuned balance between factors that activate or inhibit endothelial cell growth. About 15 proteins are known to activate endothelial cell growth and movement, including angiopoietins, epidermal growth factor, estrogen, fibroblast growth factors, prostaglandin, tumor necrosis factor, vascular endothelial growth factor (VEGF), and granulocyte stimulating factor (Zetter, *Ann. Rev. Med.* 49:407-424 (1998)). VEGF binds to tyrosine kinase receptors *flt-1* and *flk-1/KDR* on endothelial cells (Hanahan, *Science* 277(5322): 48-50 (1997)). Downstream effects of VEGF include the activation of matrix proteases and glucaronidases, loosening of endothelial cell junctions and proliferation and migration of endothelial cells. Downstream effects of basic fibroblast growth factor (bFGF) include the mitogenic stimulation of endothelial cells (Relou *et al.*, *Tissue Cell* 5:525-530 (1998)). Some of the known inhibitors of angiogenesis include angiostatin, endostatin, interferons, interleukin 1, interleukin 12, retinoic acid, and tissue inhibitors of metalloproteinase 1 and 2 (Zetter, *supra*).

C. Angiogenesis Inhibition

[0071] Because angiogenesis is essential for solid tumor growth, inhibition of angiogenesis is one strategy for preventing tumor growth. By blocking the development of new blood vessels, a tumor's supply of oxygen and nutrients can be cut off and, therefore, the tumors' continued growth and metastasis can be arrested. Several strategies can be to design anti-angiogenesis agents including: 1) blocking the factors that stimulate the formation of blood vessels; 2) using natural inhibitors of angiogenesis; 3) blocking molecules that allow newly forming blood vessels to invade surrounding tissue; and 4) incapacitating newly dividing endothelial cells. In general, tumors with higher densities of blood vessels are more likely to metastasize and are correlated with poor clinical outcomes. Also, cell shedding from the primary tumor begins only after the tumor has a full network of blood vessels. In addition, both angiogenesis and metastasis require matrix metalloproteinases, enzymes that break down the surrounding tissue and the extracellular matrix during blood vessel and tumor invasion. Several differences between standard chemotherapy and anti-angiogenesis therapy result from the fact that angiogenic inhibitors target dividing endothelial cells rather than tumor cells. Anti-angiogenic drugs are not likely to cause bone marrow suppression, gastrointestinal symptoms, or hair loss, characteristics of standard chemotherapy treatments. Also, because anti-angiogenic drugs may not necessarily kill tumors, but rather hold them in check indefinitely, the endpoint of early clinical trials may be different than for standard therapies. Rather than looking only for tumor response, it may be appropriate to evaluate increases in survival and or time to disease progression.

[0072] Drug resistance is a major problem with chemotherapy agents because most cancer cells are genetically unstable and therefore prone to mutations. Because angiogenic drugs target normal endothelial cells, which are not genetically unstable, drug resistance may not develop. So far, resistance has not been a major problem in long term animal studies or in clinical trials of potential therapeutic drug candidates. Anti-angiogenic therapy may prove useful in combination with therapy directly aimed at tumor cells. Because each therapy is aimed at different cellular targets, such combination therapy should more effective. There is growing recognition that cancer may become a chronic disease. If treatments are long term, the toxicity profile of drugs, which can be examined readily in the transparent teleost (*e.g.*, zebrafish) embryo, will become an increasingly important parameter for drug screening and evaluation.

D. Angiogenesis Stimulation

[0073] Although ischemic tissue in the heart or limbs secretes VEGF and bFGF, which stimulate local growth of collateral blood vessel, natural formation of collateral vessels feeding into ischemic tissue is rarely sufficient for full restoration of blood flow in cardiovascular disease patients. Growth of new blood vessels, induced by exogenous angiogenic agents, may restore blood flow to ischemic tissue in patients with various cardiovascular diseases. Stimulatory angiogenic therapy may also provide a minimally invasive approach to improved circulatory function in coronary artery disease (CAD), congestive heart failure, peripheral arterial disease (PAD) and peripheral venous disease (PVD). Stimulatory angiogenic therapies may also facilitate transplant acceptance or survival. Disadvantages of angiogenic stimulators include exacerbation of growth of occult tumors and progression of diabetic retinopathy. An ideal angiogenic agent for inducing growth of collateral arteries around an atherosclerotic plaque should function only in the locality of or be delivered locally to ischemic tissue.

[0074] Angiogenesis gene therapy is an experimental technique being used to trick the heart into performing its own bypass operation by growing new blood vessels. The gene coding for a protein, such as VEGF, which encourages new blood vessels to sprout from existing ones is injected into the heart of the patient and the body performs its own coronary bypass. These new vessels are less inclined to silt up again. In preliminary experiments with rabbits, the arteries in rabbit legs have been tied off and the VEGF gene has been applied directly onto the smooth muscle cells lining the artery using a catheter and small balloon. Within three to 10 days, new blood vessels were observed to sprout and find their way around the blockage. Rivard *et al.*, *Circulation* 99(1):111-120 (1999). In preliminary experiments with humans, the gene has been injected directly into the left ventricle, the pumping chamber of the heart. Results to date from these studies are promising. There have been no side effects and the worst result to date has been no result. The sprouting of new vessels, if it occurs, seems to stop after four to six weeks. Losordo *et al.*, *Circulation* 98(25): 2800-2804 (1998).

E. Angiogenesis in Zebrafish

[0075] In the zebrafish, as in other vertebrates, blood vessels form from precursors cells (angioblasts) distributed widely throughout the mesoderm of the embryo. Some angioblasts migrate long distances, while others remain locally to form vessels (Fouquet *et al.*, *supra*). The major vessels, including the aorta, vena cava, and vessels directed to some organs, are believed to form by local assembly of angioblasts into tubes (vasculargenesis). *See* Fig. 1. In addition to vasculargenesis, smaller vessels extend from larger vessels to penetrate a specific tissue (angiogenesis) (Fouquet

et al., *supra*). Experiments suggest that both processes of vessel formation - vasculargenesis and angiogenesis — are driven by local signals. By day three of development, the zebrafish has developed an intact, functioning vasculature, including both major vessels and sprouts, which has a consistent pattern of vessel location. *See* Figs. 2A and 3A. Because the zebrafish embryo can survive and develop for at least 4-5 days without a circulatory system, with the transparent zebrafish it is possible to study the effects of a variety of agents on all aspects of vascular formation in an intact, live animal.

F. Advantages of Using Zebrafish in Screening Assays for Angiogenesis

[0076] Currently, a variety of assays are used to study the process of angiogenesis in various animal models. These assays include preparing a transparent window in the skin of a rabbit or mouse, injecting tumor cells or carrier matrix into an avascular region, such as the cornea, and inducing ischemia by constricting existing blood vessels (Jain *et al.*, *Nat. Med.* 11:1203-1208 (1997)). While these and other approaches generate a great deal of information about the process of angiogenesis, the tissue manipulation required for each assay make them unsuitable for use as screening tools. (Comparative assays are further described in detail below.) Teleosts and zebrafish in particular offer significant advantages for *in vivo* screening assays for angiogenesis. As noted above, zebrafish are comparatively inexpensive to generate and maintain and the embryos can be placed in individual microtiter wells, making automated analysis with standard liquid handling equipment possible.

[0077] In addition, with teleosts, such as zebrafish, the side effects of an agent can be monitored and assessed simultaneously along with the principal effect of the agent. This provides a significant advantage in methods for screening compounds for angiogenesis activity. Notably, one difficulty associated with identifying compounds that can be used as anti-angiogenic agents, such as anti-cancer therapeutics, is that many of the compounds used to inhibit the proliferation of cancer cells also have deleterious effects on proliferating non-cancer cells. This is especially problematic when dealing with cancers that affect children, because many of their organs and tissues are still growing and developing. Using transparent teleost embryos, the effect of an agent on angiogenesis activity as well as any toxic or side effects can be assayed simultaneously. Side effects or toxic effects of agents on zebrafish cells and/or embryogenesis can be monitored at time intervals after administration of the agent. Typically, measurements are performed at the same time as measurements to assess activity of administered agents.

G. Angiogenesis Screening Methods

[0078] The present invention provides methods of screening an agent for an ability or capacity of an agent to enhance, inhibit, or block angiogenesis activity in a teleost in response to the administration of a dose of an agent to the teleost. Angiogenesis activity is assessed relative to contemporaneous and/or historical control teleosts (or tissues, organs, or cells thereof) to which the agent has not been administered. - Angiogenesis activity is reflected in changes in the vasculature of the teleost. Blood vessel formation and development can be monitored over time in the teleost to which an agent has been administered as well as in control teleosts. A response showing an increase in normal blood vessel formation suggests that the compound enhances or increases angiogenesis. A response showing a decrease or reduction in normal blood vessel formation or the death or loss of previously established, existing blood vessels suggests that the compound decreases, prevents, or inhibits angiogenesis activity (*i.e.*, enhances or stimulates anti-angiogenesis activity) or disrupts existing vessels. Responses indicating an angiogenic activity can be detected in a whole teleost or in one or more organs or tissues of a teleost, either simultaneously or separately. Responses can be detected over time and at predetermined time intervals. These responses can also be detected *in vitro* in cells of a teleost.

[0079] The methods of the present invention are useful in identifying agents that would be effective in therapeutic or prophylactic treatment of a variety of diseases involving angiogenic processes, including cancer, coronary artery disease, congestive heart failure, peripheral arterial disease, peripheral venous disease, neurological diseases, cardiopulmonary diseases, ischemia, developmental diseases, autoimmune diseases, and diseases of bone and cartilage. In general, these methods are useful in screening compounds for therapeutic activity against diseases that would benefit from an increase in angiogenesis activity (*e.g.*, increase in blood vessel formation) or decrease in angiogenesis activity (*i.e.*, anti-angiogenesis activity, a reduction in blood vessel formation).

[0080] In one aspect, the methods comprise administering the compound to be screened to a teleost embryo by submerging the embryo in culture media in which the compound has been dissolved prior to the onset of vasculargenesis or angiogenesis. After a suitable period (*e.g.*, 24 or 48 hours), the embryos are fixed and stained for an endogenous blood vessel marker, such as, *e.g.*, alkaline phosphatase (AP). A reduction or increase in the formation of blood vessels and any perturbation in the normal pattern of blood vessels can be determined visually by light microscopy after, *e.g.*, alkaline phosphatase staining, antibody staining of a protein, *in situ* hybridization. Organ or tissue function can also be determined by measuring enzymatic activity.

[0081] Compounds comprising small molecules typically penetrate the teleost embryos by simple diffusion. For com-

pounds that do not penetrate the periderm (the outer ectoderm), dimethyl sulfoxide (DMSO) or other solvents or osmotic shock can be used to transiently premeabilize the periderm. Compounds can also be administered by other well-known methods of administration, including ingestion or direct injection into either the embryo yolk or the heart of the teleost embryo. Once inside the embryo, compounds diffuse freely within the embryo.

[0082] For example, to screen for an effect of the compounds on angiogenesis activity, the subintestinal and intersomitic vessels are typically examined. To screen for an effect of the compounds on vasculargenesis activity, the dorsal aorta and ventral vessels are examined. All of these vessels are quite prominent in the unaffected teleost embryo and thus serve as ideal indicators of changes in the vascular pattern. In particular, these vessels are examined for: 1) the presence or absence of vessels, which is indicative of inhibition of angiogenesis; 2) excessive branching, which is indicative of enhancement of angiogenesis; and 3) changes in architecture of the blood vessel formation, which is indicative of changes in local signaling events. In our methods, the zebrafish embryo is used because it can survive and develop for about 4-5 days without a circulatory system and thus the effects of agents on all aspects of vascular formation in the intact embryo can be readily evaluated.

[0083] Changes in vascular pattern can be studied by performing RNA *in situ* hybridization analysis, to examine the angioblasts and vascular growth factors, and microangiography, to examine the circulation and heart function - all of which have roles in blood vessel formation. As an example, a compound to be screened is administered to a 24-hour teleost embryo by dissolving the compound in the culture medium containing the embryo in culture (prior to the onset of vasculargenesis or angiogenesis). After an additional 24 hours (at 48 hours of development), the embryo is visually inspected for morphological defects. 50% of the embryos are fixed for *in situ* hybridization using the *flk-1* probe to identify angioblasts. The remaining embryos are fixed at 72 hours of development and stained with AP. Compounds that affect the expression of endogenous AP, thereby making it difficult to assay vascular pattern by using AP staining, can be assayed by using microangiography. The embryos are then examined for any perturbation in the normal pattern of blood vessels.

[0084] Angiogenesis activity can also be detected by standard techniques indicated previously, including, *e.g.*, colorimetry, fluorescence microscopy (including, *e.g.*, time-resolved fluorometry), chemiluminescence, digital image analyzing, standard microplate reader techniques, pattern recognition software for response discrimination and analysis, *etc.* Antibody staining of specific epitopes can also be used to detect spatial or temporal changes in distribution and expression of epitopes in teleost tissues, as well as molecular modifications.

H. Screening Agents for Angiogenesis Activity And/or Toxic Activity and/or Cell Death Activity Simultaneously

[0085] The methods for screening agents for angiogenesis or anti-angiogenesis activity can be combined with other methods of the present invention described below, including methods of screening agents for an effect on cell death activity (Section III) or toxic activity (Section IV). Because the teleosts used with these methods are transparent, it is possible to assess angiogenesis or anti-angiogenesis activity in conjunction with other activities. Responses indicating various activities can also be detected in conjunction with one another - either at separate times or simultaneously.

[0086] Such combined methods are useful in assessing multiple affects of an agent on a teleost. The agent may cause both a desired response, such as enhancement of angiogenesis, and a toxic (undesired) response. The ability to assess multiple activities and responses in a teleost due to the administration of an agent is of particular benefit in identifying potential therapeutic compounds and assessing their side effects. For example, one difficulty associated with identifying compounds that can be used as anti-cancer therapeutics against targeted cancer cells is that some compounds may also have deleterious effects on non-cancer cells. Anti-angiogenic cancer therapy, for example, typically seeks to induce apoptosis in cancer cells by cutting off the blood supply of such cells. This type of treatment regime may be designed to induce apoptosis in the angioblasts as a means of preventing or diminishing vascularization of the tumor. During treatment, a balance must be achieved such that a negligible level of cell death is induced in other tissues or locations in the body (such as the heart). Such undesired ectopic cell death could be considered a toxic activity. A combination screen for assessing angiogenesis, cell death, and toxic activities of an agent would be useful in identifying those agents that protect the heart from agents which induce apoptosis elsewhere. Dose levels of the agent effective to promote one activity without promoting the other can also be ascertained. Such combined screens would also be useful in identifying and evaluating agents for pro-angiogenic therapies which typically have the therapeutic goal of preventing cell death in a damaged or transplanted tissue.

[0087] Multiple activities/responses can be monitored in the whole teleost or in one or more tissues or organs of the teleost. Such activities and responses can be monitored over time and at predetermined time intervals. A variety of techniques can be used together or separately to analyze multiple activities and responses, including, *e.g.*, fluorescence microscopy, light microscopy, digital image analyzing, standard microplate reader techniques (colorimetry, fluorometry, including time-resolved fluorometry, and chemiluminescence), *in situ* hybridization, antibody staining of specific proteins, changes in protein distribution temporally and spatially within the animal, changes in a level of enzymatic activity in the whole teleost, or tissues, organs or cells of the teleost, *etc.* Furthermore, the response can be discriminated and/

or analyzed by using pattern recognition software.

[0088] In one aspect, the present invention provides a method of screening an agent for an increase or decrease in angiogenesis activity as described above which further comprises screening the agent for an increase or decrease in toxicity by detecting a response in the teleost indicating an increase or decrease in toxic activity. Such a method is useful, *e.g.*, in identifying contra indications to therapeutic value of a compound.

[0089] In another aspect, the invention provides a method of screening an agent for an increase or decrease in angiogenesis activity as described above which further comprises screening the agent for an ability to enhance or inhibit cell death activity by detecting a response in the teleost indicating an enhancement or inhibition of cell death activity. Such a method is useful, for example, in identifying contra indications to therapeutic value of a compound. Such combination screens also allow for the identification of agents which protect the heart from circulating agents which induce apoptosis elsewhere.

Examples

1. Screening Compounds for Angiogenesis Activity in Zebrafish

A. Materials and Methods

1) Embryo Collection

[0090] Zebrafish embryos were generated by natural pair-wise mating as described in Westerfield, *supra*, which is incorporated herein by reference in its entirety for all purposes. Four to five zebrafish pairs were set up for each mating; on average, 100-150 embryos per pair were generated. Embryos were collected and placed in egg culture media prepared by combining 5 grams (g) of Instant Ocean Salt with 3 g of calcium sulfate in 25 liters of distilled water at 27°C for approximately 20 hours (21 somite stage) before being sorted for viability, using both morphology and developmental stage as criteria. Healthy embryos were then dechorionated by enzymatic digestion using 1 mg/ml protease (Sigma Chemistry Co.) for 5 minutes at room temperature. The embryos were then washed 5 times in embryo water. Because the fish embryo receives nourishment from an attached yolk ball, no additional maintenance was required.

2) Compounds Screened

[0091] Compounds from the following two sources were screened for an ability or capacity to enhance or inhibit angiogenesis activity: NCI Open Synthetic Compound Collection library, Bethesda, Maryland and The Center for Cancer Research, Massachusetts Institute of Technology (MIT), Cambridge, Massachusetts.

[0092] The NCI Open Synthetic Compound Collection library consists of more than 100,000 unique compound structures; currently, only 12,000 are available for screening.

[0093] Compounds obtained from MIT consisted of 11 fumagillin derivatives, including TNP 470 (Turk *et al.*, *Bioorg. Med. Chem.* 8:1163-1169 (1998)) and AGM-1470. Fumagillin is a natural product isolated from fungus with potent anti-angiogenic and toxic effects. AGM-1470 and the other fumagillin derivatives are angiogenesis inhibitors, which prevent entry of normal, but not transformed, endothelial cells into the G1 phase of the cell cycle by binding type 2 methionine aminopeptidase (MetAP2). The derivatives were supplied at an initial concentration of 20 mM. Samples were diluted in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) to a stock concentration of 10 mM.

[0094] Compounds from NCI were randomly selected from the NCI Open Synthetic Compound Collection library. The compounds were supplied by NCI in 96 microplate arrays, each at an initial concentration of 10 mM in DMSO. No specific information on compound source, activity, chemical structure, or mechanism of action was available.

3) Administration of Compounds

[0095] To determine the effect(s) of a compound on vessel formation on a fish, the compound was added directly to the culture medium solution containing the fish embryos (*e.g.*, to individual microwells containing the fish embryos). Compounds were added to the medium solution at 12 or 24 hours of development of the fish embryo, which is prior to the point at which angiogenic vessels can first be identified using the *flk-1 in situ* hybridization probe. Fouquet *et al.*, *supra*. Assays were performed in 6-well, 24-well, or 96-well plates. Such plates facilitated automation of the chemical application and subsequent analysis, including dose response, and subsequent analysis.

4) Visual Screening

[0096] After administering a compound to the fish embryos, the embryos were maintained in individual microwells

at 28°C until day 3 of development. Twenty-four and forty-eight hours after adding the compound to the medium in which the fish embryos were cultured, the embryos were visually inspected for viability, gross morphological defects, heart rate, and circulation (*see* Table 1). Circulation was assayed by following the movement of blood cells through each embryo.

5) Vessel Staining

[0097] On the third day of development, embryos were collected for alkaline phosphatase staining. Specifically, embryos were fixed in 4% paraformaldehyde and stained for endogenous alkaline phosphatase activity. Embryos were fixed for 2 hours at room temperature. The embryos were then washed two times in phosphate buffered saline (PBS) and dehydrated by immersion in 25%, 50%, 75% and 100% methanol in phosphate buffered saline with 0.1% Tween (PBT) to permeabilize the embryos. The embryos were then rehydrated and washed in 100% PBT. For staining, embryos were equilibrated in NTMT buffer (0.1M Tris-HCl pH 9.5; 50 mM MgCl; 0.1M NaCl; 0.1% Tween 20) at room temperature. After the embryos equilibrated, embryos were stained by adding 4.5 μl of 75 mg/ml nitro blue tetrazolium (NBT) and 3.5 μL of 50 mg/ml X-phosphate per ml. After staining for 10 minutes, all the blood vessels in the fish embryo were labeled (*see* Figs. 2A-2C, 3A-3B, 4, 7). The staining reaction was stopped by addition of PBST. Embryos were then examined on a stereo-dissecting microscope. One advantage of using the zebrafish for this type of assay is that the subintestinal vessels, which are located over the yolk, are both sensitive to factors which effect vessel formation and easily assayed by this method (*see, e.g.,* Fig. 7). The subintestinal vessels are normally present on the dorsolateral surface of the yolk of zebrafish embryos by 48 hours of development. They form a distinct basket shape that extends 50-100 μm from the ventral edge of the somite over the yolk. By assaying the subintestinal vessels at 72 hours of development (24 hours after the subintestinal vessels normally appear), normal variation in the timing of the vessel formation was avoided. The staining procedure is easily automated using commercially available instrumentation.

6) Bleaching Teleosts

[0098] If desired, teleosts (*e.g.*, zebrafish embryos) can be bleached before or after alkaline phosphatase staining. Bleaching removes the melanin pigment from the teleost and permits the screening of teleost without the adverse effects of 1-phenyl-2-thiourea (PTU) treatment. Post-stain bleaching also removes the extracellular staining associated with background staining. Bleaching effectively enhances visualization and analysis of the response of the treated teleost to a compound through the removal pigmentation of some cells. Bleaching enhances visual detection of responses indicating toxic, angiogenic, and cell death activities.

[0099] To bleach zebrafish, the fish were immersed for 10 minutes at room temperature in 5% formamide, 1X sodium chloride/sodium citrate and 10 % hydrogen peroxide.

7) *In Situ* Hybridization

[0100] In addition to performing visual screens, specific molecular changes in teleost tissues can be detected by *in situ* hybridization of RNA or antibody staining of specific proteins. *In situ* hybridization of RNA is a routine molecular approach in zebrafish (Westerfield, *supra*). A digoxigenin-labeling kit from Boehringer Mannheim can be used to label the RNA probes. Whole mount *in situ* hybridization can be carried out as follows: Embryos are fixed with 4% paraformaldehyde in PBS, lightly digested with proteinase K, and hybridized with 1 μg of probe in *in situ* hybridization solution (50% formamide, 5X SSC, 50 μg/ml Heparin, 500 μg/ml tRNA, 92 μl of 1M citric acid, pH 6.0, and 0.1% Tween 20) at 65°C. Alkaline phosphatase-conjugated anti-digoxigenin antibody is used to detect signals. Background staining from endogenous alkaline phosphatase does not pose a problem, because endogenous alkaline phosphatase does not survive the *in situ* hybridization procedure. After staining with NBT/X-phosphatase (Boehringer Mannheim), embryos are bleached in 100% methanol, refixed in 4% paraformaldehyde, and stored in PBS. Multiple *in situ* hybridizations can be performed simultaneously on different teleosts in multi-well dishes.

8) Additional Assays for Angiogenesis

[0101] To determine if any changes in vascular pattern are due to inhibition or stimulation of the angioblasts, RNA *in situ* hybridization analysis on known angioblast markers, *flk-1, tie, tek*, and *fli* (Dumont *et al.*, *Dev. Dyn.* 203:80-92 (1995); Liao *et al.*, *Dev. Suppl.* 124:381-389 (1996); Fouquet *et al.*, *supra*) can be performed using procedures outlined above. *Flk-1* (Fig. 8), *tie,* and *tek* are receptor tyrosine kinases, which label angioblasts early in development. *Fli* is a transcription factor which labels them at a later stage. Because *flk-1*, *tie, tek*, and *fli* appear sequentially during angioblast development in vertebrates (Dumont *et al.*, *supra*), assaying for the presence or absence of these molecules makes it possible not only to determine if the angioblasts are affected, but also the stage of development at which they are

affected.

[0102] Changes in the distribution of a protein both spatially and temporally, including a complete absence of a protein, within the intact teleost can be detected. For example, changes in the pattern of the vascular endothelial growth factor, VEGF, can be examined using standard antibody staining procedures (Westerfield, *supra*) or *in situ* hybridization techniques described above (*see also* Westerfield, *supra*). VEGF is believed to have two roles in vascular development: 1) a chemo-attractant or guidance role; and 2) a maintenance role (Dumont *et al.*, *supra*). Thus, chemicals which affect VEGF expression are of particular interest. The above are examples of well known molecular markers; other molecular markers can also be employed.

9) Function Assay

[0103] In addition to changes in the vascular architecture, vascular function (circulation and heart rate) may also be affected by compounds. To determine whether a compound administered to zebrafish affected vascular functioning (*e.g.*, heart rate and circulation), heart rate and circulation of the zebrafish embryos are studied. In this instance, heart rate was assessed by counting the heart beats/minute. Circulation was assessed by examining zebrafish embryos under a dissecting microscope for the movement of blood cells through the heart and major vessels. Zebrafish embryos were also examined for blood pooling in the yolk (an indicator of poor blood flow through the heart) and in the body of the embryo (an indication of leaky vessels). In those embryos in which a compound was observed to affect blood cell development, micro-angiography was performed using the procedures outlined in Weinstein *et al.*, *Nature Med.* 1: 1143-1147 (1995) to examine the integrity of the vascular system for vessel leakage and blockage, which can cause changes in vessel formation and maintenance. Embryos were anesthetized with tricaine to stop the heart, a micropipet was inserted into the heart, and fluorescent beads were injected. The tricaine was then washed out, and the heart resumed beating. The flow of fluorescent beads was then observed using an epifluorescence microscope and recorded using a low light level camera attached to a computer (Fig. 9). This approach allows examination of the integrity of the vascular system and assessment of the effects of the chemicals on the condition of the heart.

B. Results

1) Determination of Parameters for the Delivery of Compounds to Target Tissues and Organs

a) Embryo Developmental Stage

[0104] In our initial studies, we employed 12-hour zebrafish embryos (6 somite stage) for the assays and began the assays at the 12th hour of development. Although this time point is advantageous because it is just prior to the onset of angioblast formation (Fouquet *et al.*, *supra*), there are several disadvantages. The most significant of these is that at 12 hours of development, many structures of the zebrafish embryo including the notochord, the somites, and the heart are beginning to form. Because these structures directly affect both vasculargenesis and angiogenesis, it is difficult to determine if the observed effects of compounds on vessel formation are primary (direct effects on the vessels) or secondary (indirect effects due to damage to other tissues).

[0105] To circumvent this problem, we began the assays at 22 hours of development (26 somite stage). At this stage of development, the dorsal aorta and ventral vein are present in the anterior, but not in the posterior regions of the zebrafish embryo. This permitted examination of both vasculargenesis and angiogenesis independently in the same embryo. For vasculargenesis, we examined the embryos for the presence of the dorsal aorta and ventral vessel in the most posterior regions of the tail. For angiogenesis, we examined the embryos for the presence of sprouting vessels, including the subintestinal and the intersomitic vessels. The subintestinal vessels begin to form at 36 hours of development; therefore, using the 22-hour time point reduces the time between compound administration and angiogenic vessel formation. This is an important consideration for compounds that are unstable under the culture conditions.

b) Embryo Maintenance

[0106] Initial experiments were performed in 35 mm wells in 6-well culture dishes using 50 zebrafish embryos per well in 5 ml of embryo water. While this approach worked, it has a number of drawbacks, including that: 1) a relatively large amount of compound must be used to dose the embryos; 2) the number of compounds that can be screened simultaneously is limited; and 3) because there are multiple embryos in a dish, dying embryos could contaminate living embryos.

[0107] In an attempt to circumvent these drawbacks, we examined two alternative formats, the 96- and the 24-well plate. Previous observations indicated that single zebrafish embryos-were capable of surviving and developing normally in 50-100 µl of embryo water for up to 5 days. Therefore, we collected, dechorionated and sorted 22 hour embryos

into either: 1) 96 well plates with one embryo per well in 100 μl; or 2) 24 well plates with 5 embryos in 500 μl of embryo water. The embryos were allowed to develop for 72 hours before examination. The embryos were assessed by size, morphology, and movement. No obvious differences were observed between the embryos raised in the microwell plates and control embryos raised in larger containers. The embryos were fixed and stained for endogenous alkaline phosphatase to examine vessel formation. The staining pattern in the experimental embryos was identical to that observed in the controls. For the manual screen, we preferred the 24 well format and used it for all experiments described below.

c) Compound Delivery

[0108] In order to optimize the parameters for screening compounds, we performed a series of experiments using the 11 fumagillin derivatives obtained from MIT and 10 random compounds obtained from the NCI Open Synthetic Compound Collection library. We knew from our feasibility studies that fumagillin inhibited angiogenesis in the zebrafish; we thus decided to use these compounds as positive controls to verify the assay. We also used the 10 compounds from NCI to verify that the established parameters were appropriate for other types of compounds. In general, we expected that the small molecules would diffuse freely both into the embryo and through the chorion membrane that surrounds the embryo for the first 2-3 days of development. However, to avoid potential problems, we removed the chorion by enzymatic digestion. This approach is well established and when done properly produces no adverse effects on the embryos.

Table 1.

Summary of Concentration Effects of Compounds						
Compound	**100 μM**	**Effect**	**10 μM**	**Effect**	**1 μM**	**Effect**
MIT (11)	11/11	Lethal	7/10	Vascular effects Developmental Delay	11/11	Slight Developmental Delay
NCI (10)	4/10 6/10	Lethal Slight Developmental Delay	1/10 2/10 10/10	Lethal Chromatic Change Slight Developmental Delay	1/10 10/10	Lethal Slight Developmental Delay

d) Compound Concentration

[0109] As a primary screen for compound effects, we tested each compound at three different concentrations to determine which concentration would provide the most information. The concentrations tested were 100 μM, 10 μM, and 1 μM. Results are summarized in Table 1. For these experiments, we added 50 μl of 10 mM stock solution to 5 ml of embryo water to generate a 100 μM solution in 1% DMSO. The subsequent concentrations were generated by 1:10 and 1:100 dilutions in embryo water; for each concentration, DMSO added to 1% of the total solution. The control solutions consisted of 1% DMSO in embryo water. Ten embryos per compound per concentration were tested. Of the 21 compounds tested, 15 (11/11 MIT, 4/10 NCI) were lethal at the 100 μM concentration. At the 10 μM concentration, 7/11 of the fumagillin derivatives had an inhibitory effect on angiogenesis. However, while none of the fumagillin derivatives were lethal at 10 μM, they all had a deleterious effect on the growth of the embryo (Fig. 2A), consistent with previously published results showing that the target of fumagillin derivatives is methionine aminopeptidase (type 2), which plays a role in cell cycle control in eukaryotic cells (Ishikawa *et al., J. Exp. Ther. Oncol.* 6:390-396 (1996); Kria *et al., Curr. Eye Res.* 10:986-993 (1998).

[0110] In contrast to the fumagillin derivatives, at 10 μM the NCI compounds had no observable effect on vessel formation. However, 1 of the 10 NCI compounds was lethal at this concentration and 2 of the 10 compounds caused a chromatic change in the embryos. The chromatic changes were not limited simply to taking up the color of the compound; one of the NCI compounds caused the melanocytes to turn purple. As with the fumagillin derivatives, all 9 of the non-lethal NCI compounds caused a slight developmental delay, because the embryos appeared by morphological criteria to be ~12 hours delayed in development. At 1 μM, 20/21 compounds caused developmental delay and 1/21 caused lethality. These results show quite clearly that compounds added to the media were capable of getting into the zebrafish embryo and inducing an effect.

e) Use of DMSO

[0111] One problem with the experimental conditions described above was that the control embryos maintained in 1% DMSO in embryo water also showed a slight developmental delay, similar to that observed for all of the concentrations of the NCI compounds and for the 1 μM concentration the fumagillin derivatives. We repeated the experiments using 10 μM and 1 μM concentrations of the compounds, respectively, in 0.1% DMSO. The results were identical to those in Table 1, except that the developmental delay for all of the compounds except the fumagillin derivatives at 10 μM concentration was eliminated. After performing these experiments, we decided to use 10 μM concentrations with 0.1% DMSO. The results indicated that at relatively high concentrations, DMSO has some effect on developing zebrafish. While DMSO does not appear to have any effect on developing zebrafish at lower concentrations, we are aware that synergistic effects may occur. Unfortunately, many of the compounds available for screening were only soluble in DSMO or similar solvents. As with any primary screen, positive results will require further verification and scrutiny.

2) Assessing the Effects of Compounds on Blood Vessel Formation

[0112] After establishing basic assay parameters, we screened compounds received from MIT (11 compounds) and NCI (190 compounds) for effects on blood vessel formation (angiogenesis and vasculargenesis). Embryos were collected at 20 hours of development and dechorionated. At 22 hours of development, the embryos were sorted into 24 well plates with 5 embryos per well in 500 μl of embryo water. The compounds from MIT and NCI were added at a concentration of 10 μM. For each compound, 3 sets of embryos (15 total) were screened. For convenience, each set was maintained in a separate multi-well plate. This permitted testing of 23 compounds/plate with 1 set of controls per plate. At 72 hours of development, embryos were visually screened for gross morphological defects and cardiac function using a dissecting microscope. After the visual screen, embryos were fixed and stained for endogenous alkaline phosphatase activity in order to analyze vascular architecture. Experimental results are shown in Table 2 and described below.

Table 2.

Results of Visual Screen							
Compounds (Compds)	**Number Compds Screened**	**Vascular Changes**	**Develop-mental Delay**	**Axial Defects**	**Cranial Defects**	**Circulation/ Heart Rate Defects**	**Toxic At 10 μM Compd**
NCI	190	18	16	6	7	6	13
MIT	11	7	11	3	0	0	0

a) Vascular Changes

[0113] To assay vessel formation, embryos were fixed and stained and the vessels were scored as described above. The subintestinal vessels form on the dorsolateral surface of the yolk on both sides of the embryo in the shape of a basket that extends 50-100 μm from the ventral edge of the somite over the yolk. For this screen, anti-angiogenic effects were defined as either the complete absence of these vessels or the loss of either the lateral or dorsalventral vessels of the basket (Figs. 2B-2C, 3B, 4). An angiogenic effect was defined for this screen as an enlargement of the basket beyond 150 μm from the somite. This includes both increases in size of the entire basket and/or projections from the basket (Figs. 6A, 6C, 6D). In addition to the overall basket size, we also looked for increases in the diameter of the vessels. Normal vessels are less than 10 μm in diameter. Embryos were also screened for gross changes in the large vessels, including the dorsal aorta and ventral vein.

[0114] Of the 241 compounds tested, 25 (7/11 from MIT and 18/190 from NCI) caused some anti-angiogenic effects (Table 3). Of these, 23/25 were associated with various degrees of developmental delay; the more severe the delay, the more dramatic was the reduction in vessel formation (Figs. 2A-2B). Of the two other compounds that caused a reduction or loss in vessel formation, one was associated with a truncation of the embryonic axis (Fig. 4). Axial defects do not generally cause a loss of the subintestinal vessels, suggesting that the vessel effect may be distinct from the axial effect. Only I of the compounds tested showed a specific effect on vessel formation. With this compound, there was a loss of the subintestinal vessels (Fig. 2B), with no other observable effects on the embryo.

Table 3.

Observed Effects of Compounds on Vessel Formation						
Compds (Source)	**Vessel Effect**	**Complete Loss of Subintestinal Vessels (SIVs)**	**Loss of Lateral or Dorsalventral Vessels of SIVs**	**Increase in SIVs**	**Increase in Vessel Diameter**	**Changes in Large Vessels**
MIT (11)	7/11	4	3	0	0	5
NCI (190)	18/190	5	13	0	0	3

[0115] With 8/25 compounds that caused angiogenic effect, we observed what appeared to be a failure of the large vessels to organize properly in the tail. We only observed this effect with compounds that induced severe developmental delay. It is likely that the failure of the aortal and ventral vein to organize properly may be a secondary effect. Curiously, none of the compounds screened caused an increase in vessel formation as assayed by increases either in size of the subintestinal vessels or the diameter of the vessels.

[0116] To determine if the loss of the subintestinal vessels was due to the loss of angioblast, we performed an *in situ* analysis on embryos treated with the compounds that had previously been shown to cause a reduction of vessel formation. We used a probe against *flk-1*, a receptor tyrosine kinase that has been shown to play a pivotal role in angiogenic vessel formation (Hanahan, *supra*). *Flk-1* has been shown to be the earliest marker for angioblast in the zebrafish embryo (Fouquet *et al.*, *supra*). Normally, *flk-1* is highly expressed throughout development in newly forming vessels and expressed at lower levels in the large vessels after their formation. In the absence of angiogenic vessels, we would not expect to see *flk-1* expression in the somitic and subintestinal region; therefore, we focused on expression of *flk-1* in the large vessels.

[0117] Embryos were collected at 48 hours of development (24 hours after addition of the compounds) because *Flk-1* is still highly expressed in the large vessels at this stage. For 17 of the 18 NCI compounds which caused a reduction in the subintestinal vessels, the pattern of *flk-1* staining appeared normal at 48 hrs of development. Specifically, *flk-1* staining was present in the dorsal aorta and ventral vein as well as in the vessels of the head. There was no staining in either the intersomitic space or on the dorsal surface of the yolk, however; this was expected, because these vessels did not form. One compound caused a loss of *flk-1* staining in the large vessels of the tail, but not in the head. This compound also caused a truncation of the embryo, a thinning of the tail and heart malformation (Fig. 5). It is probable that the loss of *flk-1* staining was part of a more global defect, rather than a specific anti-angiogenic effect.

b) Developmental Delay

[0118] Because a number of the defects included changes in the size and shape of the embryo, to distinguish between morphological defects and developmental delay, we used three distinct parameters. Normally, zebrafish embryos are staged by the position of the head on the yolk ball, the length of the embryo, and the position of the forming melanocytes. As our 4th criteria, we used the size and shape of the fins to assist in staging the embryos. For this screen, developmental delay was defined as at least 12 hours slower than the control embryos on the same multi-well plate. As previously noted, all the fumagillin derivatives caused a substantial developmental delay of at least 24 hours (Figs. 2A-2C). This is probably because the target of these compounds is a cell cycle regulating protein (Turk *et al.*, *supra*). Developmental delay was also observed in 16 of the 190 (8.5%) small molecule compounds from NCI. In all cases where developmental delay was observed, there was a change in vascular architecture consistent with the developmental delay (Figs. 2A-2C). It is likely that compounds that affect proliferation and growth will also affect angiogenic vessel formation, which requires cell proliferation in order to form new vessels. Eight of these 16 compounds also caused what appeared to be a disorganization of the large vessels in the tail.

c) Axial Defects

[0119] There were three typical types of axial defects: 1) bending of the axis either up or down (NCI 3/6, MIT 3/3); 2) truncation of the axis (NCI 2/6); and 3) blebbing of the notochord (Fig. 5)(NCI 2/6). A reduction of the subintestinal vessels was observed with only one of the compounds that caused an axial defect (Fig. 4).

d) Cranial Defects

[0120] Cranial defects were defined as either the disruption of the central nervous system (CNS) morphology, usually at the midbrain/hindbrain border, or the presence of cellular debris in the ventricular space of the CNS. Seven of the 190 NCI compounds caused cranial defects; however, none affected the subintestinal vessel or the large vessels in the tail.

e) Toxicity

[0121] For this specific experiment, we defined toxicity as whole embryo lethality by 72 hours of development. Using the previously established assay parameters, we predicted that the 10 μM concentration of a compound was unlikely to induce toxicity. Therefore, we were not surprised that only 5% (6.8% of NCI, 13/190) of the compounds tested were lethal. Of the 13 lethal compounds, eight killed the embryos within 24 hours of application. The remaining 5 compounds caused localized cell death (4 in the tail and 1 in the head) within 24 hours and whole embryo lethality by 72 hours of development. It is possible that at lower concentrations these compounds can affect angiogenesis without causing toxicity; however, this seems unlikely, as the toxic effects were quite global.

3) Assessing Effects on Vascular Function

a) Circulation/Heart Rate Defects

[0122] There were a number of compounds causing developmental delay and axial defects that also caused structural changes in the heart. In general, these effects were consistent with underdevelopment of the heart. In order to evaluate function, we restricted our analysis to embryos in which the heart appeared relatively normal, as defined by the presence of an atrium and a ventricle, as well as a heartbeat. 6/190 of the NCI compounds caused a reduction in the beat rate of the heart. For this screen, reduced heart rate was defined as 50% or less than the rate of controls. Because biological and environmental factors cause natural variations in the heart rate, the normal heart rate was taken as the average heart rate of the 10 embryos in the control wells for each plate. This was compared to the average heart rate of the embryos in the experimental well. In 3/6 compounds, pericardial edema and blood pooling over the yolk accompanied the reduced rate. Even though pericardial edema was evident, blood cells moved through the major vessels. All three of these compounds caused developmental delay with an associated reduction in angiogenic vessels; specifically, the subintestinal vessels were absent. The remaining 3 compounds had no observable effects other than reduced heart rate.

[0123] None of the non-lethal compounds tested caused an observable reduction in the number of blood cells; thus, it was possible to assay circulation by observing the movement of blood cells through the vessels. As with assessment of heart rate, only embryos with structurally normal hearts were analyzed, because malformed or underdeveloped hearts cannot usually pump blood. None of the compounds appeared to affect circulation as assessed by lack of blood flow, blood pooling, or leaky vessels.

[0124] Circulation was assayed by observing the flow of blood cells through the embryo. Of the 212 compounds tested in this study, none affected the formation of the blood cells; therefore, it was not necessary to perform any microangiograms to assay circulation. However, because it is unlikely that this will be the case for all compounds, the microangiogram technique is typically included as part of the screening methods. A microangiogram was performed as part of our initial studies on a zebrafish embryo at day three of development. The microangiogram shows the normal vascular pattern of the zebrafish embryo, including the cranial, intersegmental, and subintestinal vessels. *See* Fig. 9.

C. Discussion

[0125] The above results demonstrate that the teleost (*e.g.*, zebrafish) is a viable model for screening small molecules (*e.g.*, chemical compounds) for effects on vessel formation. Such small molecules not only diffuse into the embryo, but can also induce specific, observable effects on blood vessel formation.

1) Diffusion of Small Compounds into the Teleost Embryo

[0126] One major concern prior to experimentation was whether different types of small molecules would diffuse into the zebrafish embryos after addition to the media. Our initial studies demonstrated that fumagillin and ovicillin were capable of diffusing into the zebrafish embryo. However, these compounds are natural products identified because of their ability to diffuse into cells in culture. Of the 201 small molecule compounds screened, 81 had some observable effect on zebrafish embryos (70/190 compounds, including 23 which caused color changes (data not shown), from NCI

and 11/11 fumagillin derivatives). These results suggest that our initial assumption that small molecules would enter the embryos by diffusion was correct.

2) Advantages of Whole Embryo Screening

[0127] One significant advantage of using whole teleost embryos for assays is the ability to identify effects on multiple targets simultaneously. In our initial set of experiments, we restricted additional targets to events that could be visualized without additional staining.-Developmental-delay was-the most-useful of these parameters. Unlike with cell culture assays, with the whole embryo assay, we were able to observe that the 11 MIT compounds caused what appeared to be general cell proliferation effects, which may or may not be the same as anti-angiogenic effects. This may be due to the binding of type 2 methionine aminopeptidase (MetAP2)(Turk *et al.*, *supra*) or a related cell cycle protein.

[0128] We also observed a number of other effects with other compounds. With 6 compounds, we observed effects on heart rate in live embryos by visual inspection. Because the heart is quite prominent in the early embryo, it was possible to observe a slow versus normal heart rate by visual inspection. Two possible mechanisms for this observed effect are: 1) the compound may affect development of the heart in such a way that the conductivity mechanism required for normal heart beat is absent, or 2) the compound directly antagonizes the conductivity mechanism in a manner similar to beta-blockers (Reiter and Reiffel, *Am. J. Cardiol.* 82(4A):9-19 (1998)). We were also able to score cranial defects in 7/201 compounds, as well as axial defects in 9/201. In subsequent studies (described below), we used specific antibodies and staining techniques to analyze the effects of compounds on other organs, including the liver and the kidney, to determine adverse effects of angiogenic compounds. The liver and kidney are highly vascularized; as a result, these organs represent potential targets for screening compounds for adverse effects on blood vessel formation.

3) Screening for Anti-Angiogenic Effects

[0129] In our first set of screening experiments for anti-angiogenic effects, we examined the effect of fumagillin, a natural anti-angiogenesis chemical, on blood vessel formation in the zebrafish embryo. The compound was administered by addition to the fish culture media. This compound caused a reduction in angiogenesis, indicated by a reduction of the subintestinal and intersomitic vessels (*see, e.g.,* Fig. 7). However, each compound also caused serious complications in the embryo including pericardial edema, developmental delay, and axial defects. Although these experiments demonstrated the feasibility of the approach for drug screening, they also underscored the importance of identifying compounds that affect angiogenesis selectively. Using the screening parameters described above, we identified two compounds that caused apparently specific anti-angiogenic effects. In addition, we identified 16 other compounds that caused a reduction of angiogenic vessel formation in addition to other effects. These results show that the zebrafish embryo model can be used to screen for compounds that specifically affect angiogenesis and anti-angiogenesis activities.

4) Screening for Angioblast Formation Using *Flk-1* Staining

[0130] 1 of the 18 NCI compounds that caused a reduction in subintestinal vessel formation had an effect on the *flk-1* staining pattern. Because *flk-1* is an early marker for angioblasts, this result suggests that for 17/18 compounds, the blocking of angiogenesis is not due to loss of angioblast, but rather to interference with some other component of the angiogenic pathway. For the one compound that did affect *flk-1* staining, it was not clear if the loss of staining was due to a loss of angioblasts or loss of the *flk-1* tyrosine kinase expression. This demonstrates the importance of establishing markers for both angioblasts and the angiogenic pathway (see discussion below).

5) Angiogenic Effects

[0131] None of the compounds tested caused an observable increase in vessel formation. Two possible explanations of this observation are: 1) none of the compounds tested had angiogenic properties; and 2) the normal zebrafish embryo is refractory to exogenous angiogenic stimulation. To distinguish between these two possibilities, we performed experiments, described below, in which VEGF was injected into 24 hour embryos. These experiments suggested that increased angiogenesis can be induced in the normal zebrafish embryo (Figs. 5A-5C). In order to increase the likelihood of identifying compounds which stimulate angiogenesis, we explored the use of mutant zebrafish lines, such as the gridlock mutant (Weinstein *et al.*, *supra*), which has defects which block angiogenesis.

6) Vasculargenesis

[0132] In the zebrafish as in humans, vasculargenesis is the process by which the large vessels, including the aorta, vena cava, and vessels to some organs, form from local precursors cells (angioblasts) distributed throughout the mesoderm of the embryo (Fouquet *et al.*, *supra*). We observed vascular defects with 8 of the 241 compounds screened. The effects observed were limited to a disorganization of the dorsal aorta and ventral vessel in embryos with severe developmental delay. It is not clear that vasculargenesis requires-extensive cell proliferation; and these observed effects on the large vessels may be due to a disruption of the surrounding tissue, rather than a direct effect on the angioblasts.

7) Rapid and Automated Methods of Screening of Agents for Angiogenesis Activity

[0133] Our experiments demonstrated the versatility and value of the teleost as a model for use in detecting, identifying, and analyzing compounds that inhibit or enhance angiogenesis *in vivo* and *in vitro*. With methods of the present invention, teleosts (*e.g.*, zebrafish embryos) can be used to screen large numbers of compounds rapidly for effects on angiogenesis. For example, using the 24 well format and manual techniques for fluid changes, we screened 241 compounds for a variety of effects at multiple time points. These effects included morphological defects, functional defects, and lethality. While these target effects provide a tremendous amount of information, analysis of other targets such as heart rate, circulation, and other organs constitute a secondary level of screening that should be performed only on compounds pre-screened for angiogenic effects. A primary screen for compounds which affect angiogenesis should focus on the stained subintestinal vessels in 72 hour embryos.

[0134] The present invention also includes automated methods for rapid screening of compounds that enhance or inhibit angiogenesis activity in animal models *in vivo* and *in vitro in* cells thereof. Preferred animal models include transparent teleosts, such as zebrafish. Any of the compounds described herein can be screened using automated procedures described previously, including, *e.g.*, small chemical compounds or larger biological molecules discussed below.

[0135] In our analyses discussed above, we screened 190 compounds from the NCI Open Synthetic Compound Collection library. Although this library consists of more than 100,000 unique compound structures, currently only 12,000 are available for screening. Using the manual screening methods of the invention, the entire compound library can be screened in two years. Incorporation of commercially available fluid handling instrumentation significantly reduces this time frame to less than three months.

2. Screening Biological Molecules for Angiogenesis Activity

[0136] The present invention also includes methods of screening of larger molecules, including biological molecules, for an ability to enhance or inhibit angiogenesis activity. These methods comprise administering the compound to a teleost and detecting a response indicating an enhancement or inhibition in angiogenesis activity. No precise method for screening large biological molecules for angiogenesis activity currently exists. Thus, the methods of the invention are thus of particular value and use in evaluating the use of biological compounds as therapeutics and/or prophylactics for treating a variety of diseases in humans associated with angiogenesis processes, including neurological diseases, cardiopulmonary diseases, ischemia, developmental diseases, autoimmune diseases, diseases of bone and cartilage, and cancer.

[0137] A wide range of biological compounds, including peptides, proteins, glycoproteins, nucleic acids (*e.g.,* DNA and RNA), lipids, glycolipids, and the like, including, but not limited to, derivatives, analogues, and chimeras of such compounds, can be screened by these methods. As discussed above, compounds from a library of compounds, including a combinatorial library, can be screened.

[0138] Recently, a number of biological molecules have been identified that have either anti-angiogenic or angiogenic effects (Hanahan, *Science* 277(5322):48-50 (1997); Zetter, *supra*). Some biological compounds have been characterized and analyzed for angiogenesis activity in cell cultures and in mice; a few such compounds have been tested in therapeutic and/or prophylactic treatment programs in humans. Comparison of the results using these compounds and the teleost model and screening methods of the present invention would allow a determination as to whether the teleost model and screening methods described herein is predictive of the therapeutic effect in humans; such a comparison would be of benefit in determining whether a tested compound would be useful in programs for therapeutic and/or prophylactic treatment of angiogenesis-related disorders in humans.

A. Proteins

[0139] To examine the effects of proteins and protein fragments (and peptides and peptide fragments) on angiogen-

esis in teleost embryos, proteins (and protein fragments and peptide fragments) were directly injected into the circulation of 24 hour zebrafish embryos. Embryos were collected and dechorionated as described above. The embryos were then sorted into holding ramps made of 1% agarose in embryo water and oriented with the yolk ball projecting up. Microinjection injection was performed as follows: the proteins were suspended in PBS and backfilled into a pulled glass micropipet. The micropipet was then attached to a micromanipulator and a picospritzer (General Valve) attached to a nitrogen tank. Using the micromanipulator, the tip of the micropipet was inserted into the embryo and a small volume of protein solution was expelled from the tip using positive pressure. To determine if our animal model could be used effectively to screen for these compounds, we performed a series of experiments in which we injected one of two different proteins having opposing effects on vessel formation - human endostatin (O'Reilly *et al.*, *Cell* 88(2):277-285 (1997)) and human vascular endothelial growth factor (VEGF) - into an embryo. Endostatin, a collagen XVIII fragment, is an endogenous protein with potent anti-angiogenic activity. VEGF has been shown to play a critical role in both endothelial cell determination, as well as vessel formation. In preliminary experiments, we injected VEGF protein either into the yolk ball or into the perivitelline space between the yolk and the periderm. Because the second location is in the path of the venous return, proteins end up in the circulation of the embryo. To backfill the injection pipettes, we used a 2 mg/ml solution of VEGF. When VEGF was injected into the yolk, we observed two angiogenic phenotypes: 1) the appearance of long spikes projecting from the subintestinal vessel basket (Fig. 6A); and 2) increased vessel diameters in the subintestinal basket (Fig. 6C). In contrast, injections of VEGF into the perivetellin space led to a disruption of vessel formation (Fig. 6D) and heart development. This is consistent with observations in other vertebrates. Drake *et al.*, *Proc. Natl. Acad. Sci. USA* 92(17):7657-7661 (1995); Fouquet *et al.*, *supra*. Endostatin was injected into the zebrafish as was VEGF. In contrast with VEGF, endostatin results ere inconsistent and thus uninterpretable. These experiments demonstrated that changes in the vascular pattern can be induced in our animal model. Moreover, because human proteins produced these effects, these experiments suggested that the mechanisms for angiogenesis in zebrafish and humans are probably similar.

B. Nucleic Acids

[0140] To deliver nucleic acids to teleosts, we established a microinjection system. Microinjection of DNA, RNA, and proteins is a well established procedure used in a variety of biological systems, including single cells, frog embryos, mouse embryos, and zebrafish. Westerfield, *supra*. In the zebrafish, it is possible to load every cell of the embryo by injecting molecules of interest into the yolk of 1-16 cell stage embryos. *See* Westerfield, *supra*. Using these standard approaches, several hundred embryos can be loaded in a two-hour period.

3. Evaluation of Biolistic Cell Loading Technology

[0141] Biolistic cell loading technology uses coated particles to introduce molecules of interest into tissues and organs of an animal. In this technique, particles coated with the biological molecule are "biolistically" shot into the cell or tissue of interest of the animal using a high-pressure gun. This technique has been used successfully to load primary culture cells as well as whole mouse embryos with large DNA plasmid constructs. Chow *et al.*, *Amer. J. Pathol.* 2(6):1667-1679 (1998).

[0142] With the methods of the invention, biolistic cell loading can be used as an alternative to microinjection techniques to inject compounds into animals, such as, *e.g.*, adult, larval, and teleost embryos. DNA can be regionally administered to the teleost (*e.g.*, introduced to specific locations within the teleost embryo), such as the tail or the dorsal surface of the yolk, prior to, after, or at the time angiogenesis begins.

4. Establishing Parameters for Quantifying and Characterizing the Effects of Compounds on Angiogenesis Activity and Endothelial Cell Toxicity

[0143] To determine whether a particular compound is of potential therapeutic or prophylactic use, a number of additional parameters, including the Therapeutic Window and the Effective Window can be determined.

A. Therapeutic Window

[0144] The Therapeutic Window (TW) is the ratio of the Median Effective Concentration (EC50) to the Median Lethal Concentration (LC50) (*i.e.*, LC50/EC50). LC50 is determined by administering serial dilutions of an agent and determining what proportion of teleosts die at each dilution. LC50 is the concentration needed to cause lethality in 50% of the teleosts. Agents which exhibit a high Therapeutic Window (LC50/EC50), such as 100 or 1,000, are good potential drug candidates because toxicity at the therapeutic concentration is low. Agent concentrations typically range from picomolar to millimolar.

B. Effective Window

[0145] The Effective Window (EW) identifies the point during angiogenesis at which a compound is effective. This is determined by exposing embryos to the EC50 of a compound at different stages of angiogenesis, beginning with the 12 somite stage, when angioblasts are first detectable, through the 72 hour stage, when vascularization in the embryo is complete.

[0146] In our preliminary studies, we identified a number of compounds which were toxic at various concentrations. It is possible that such compounds are extremely potent and that only low (picomolar) concentrations of such compounds effect angiogenesis. This problem can be addressed by screening compounds for angiogenic effects at concentrations well below the concentration at which induces toxicity.

C. Quantitation of Vessel Growth

[0147] While visual comparison of an embryo treated with a compound of interest with an untreated embryo (control) is an effective means for identifying changes in the vessel architecture related to angiogenesis, it does not permit quantitative assessment. As an alternative or in addition to visual comparison, image analysis can be used to quantify and standardize the analysis. A number of commercially available software packages exist (*e.g.*, Image-Pro Plus™, Media Cybernetics; WSR Image Analysis System, WindSword Software Research; MetaMotph®, Universal Imaging Corp.) that permit both distance and area measurements of vessel dimensions and distribution - the parameters used for visual analysis.

D. Evaluation of Additional Markers for Characterizing Angiogenic Activity

[0148] Antibody markers that label signaling proteins involved in angiogenic vessel formation in the zebrafish, including VEGF and Ang1 and 2 (Hanahan, *Science* 277(5322):48-50 (1997)), would assist in identifying compounds that are either agonists or antagonists of the signaling molecules that guide vessel development and patterning. A number of antibody markers have been identified in mouse and are commercially available (Santa Cruz Biotechnology, Inc.). These markers can be tested in teleosts using standard antibody staining protocols. Westerfield, *supra*. Antibodies can be used in place of RNA probes to simplify the assay procedure.

[0149] Briefly, embryos are fixed for 2 hours at room temperature. The embryos are then washed two times in phosphate buffered saline with Tween (PBT) and permeabilized by treatment acetone at -20°C for 7 minutes. The embryos are rehydrated and then treated with a blocking solution (2% goat serum, 1% bovine serum albumin (BSA) in PBT) for 30 minutes at room temperature. Next, the embryos are soaked in blocking solution containing the primary antibody overnight at 4°C. The embryos are then be washed 5 times in PBT with 1% BSA. The embryos are soaked in blocking solution containing a secondary HRP-conjugated antibody for 4 hours at room temperature. The embryos are then washed and stained by soaking in DAB solution (1 mg diaminobenzidine, 1 ml 0.1M PO_4 buffer, 1 ml dH_2O and 20 µl of DMSO) for 15 minutes. H_2O_2 is then added to the solution for color development. The reaction-is stopped by adding PBT.

E. Assays for Compounds that Induce Endothelial Cell Toxicity

[0150] Although the strategy of blocking new vessel formation has significant potential for anticancer therapeutics, an alternative strategy is to destroy vessels already present in the tumor. With such a strategy, a compound is administered to the teleost after vessel formation, not prior to vessel formation. It is not known how long compounds persist in the media containing teleost embryos; we assume that effects on blood vessel formation occur relatively soon after administration of the compound. To identify compounds that have toxic effects on blood vessels after formation, we administered compounds to zebrafish embryos at 60 hours of development, when the subintestinal vessels were well established. We then assay the embryos at 72 and 84 hours of development. Compounds were screened for those which caused a loss of the subintestinal vessel staining, as described above.

F. Evaluating the Use of Mutant Fish Lines

[0151] Studies suggest that it may be difficult to induce additional vessel growth in a normal animal system. For example, there is evidence of this in the mouse model for ischemia (Couffinhal *et al.*, *Amer. J. Pathol.* 2(6):1667-1679 (1998)). This issue can be circumvented-by performing screens on animals in which vessel development has been impaired. A few genetic mutations exist in the zebrafish that disrupt vessel formation. Examples of such mutations are: 1) gridlock, alocalized heritable vascular patterning defect in the zebrafish (Weinstein *et al.*, Cardiovascular Research Center, Massachusetts General Hospital, Charleston, MA (1998)), in which vessel formation is normal in the head

region and absent in the tail for the first 3-4 days of development, and after ~4 days, collateral vessels begin to appear in the tail; 2) cloche (Fouquet *et al.*, *supra*, Thompson *et al., Dev. Biol.* 197(2):248-49 (1998)), in which angioblast development is impaired; and 3) no tail and floating head (Fouquet *et al.*, *supra*), notocord mutants, in which the formation of the large vessels is blocked. The usefulness of these and other mutant lines are readily evaluated by using the methods for screening compounds for angiogenic activity described herein. Currently, there are no known mutations that cause increases in vessel growth in zebrafish.

G. Assessing Effects of Angiogenic/Anti-Angiogenic Compounds on Organ Systems

[0152] The effects of a compound on organ systems other than the vascular system (*e.g.*, kidney, heart, *etc.*) can be determined by using screening methods described herein. The ability to make such determinations is significant, because in evaluating the potential therapeutic value of any compound identified using the methods of screening compounds for angiogenesis activity, it is important also to identify adverse effects, including adverse effects on other organ systems. The teleost model is ideal for this purpose because many of its organs can be visualized in the transparent teleost embryo by light microscopy (*e.g.*, the heart and the CNS); alternatively, a number of organs of the teleost embryo can be identified by simple staining techniques (*e.g.*, liver, gut, heart, and kidney). For example, cardiac function and liver viability can be assayed. Because the heart is both directly connected to the vasculature and because the heart and the vessels share some of the same cell types, the heart is a likely secondary target of compounds that affect angiogenesis activity. Because the liver is the site of accumulation and metabolism of many compounds, especially toxins, it is an indicator of the toxicity of both the compounds and the breakdown products.

[0153] As described above, in our initial studies, we observed that a small number of compounds affected the heart rate of the zebrafish embryo. With six compounds, we observed that the zebrafish heart beat at approximately 1-2 beat (s) per second instead of the normal 4-5 beats per second. To determine if a particular compound affected teleost heart development, or if it acted as an antagonist to the conductivity mechanism, we administered the compound of interest to a zebrafish embryo at 72 hours of development, when a functioning heart and vascular system is present. The embryos were then evaluated 2 hours after the addition of the compound for immediate effects on heart rate and contractility and at 24 hours for effects which might require novel gene or protein expression. If a compound acted as a direct antagonist on either the conduction or contraction machinery of the zebrafish heart, its administration to the zebrafish at any stage would likely show an effect. However, if a compound affected development of the zebrafish heart, its presence should show no effect at the later stage of development.

[0154] In addition to examining the heart rate and contractility, we also examined the structure of the heart both by visual inspection (Stainier *et al., Development* 123:285-92 (1996)) and by staining the heart with antibodies against tropomyosin and cardiac myosin heavy chain (Stainier and Fishman, *Trends Cardiovasc. Med.* 4:207-212 (1994), which allowed identification of the atrium and the ventricle - the two chambers of the fish heart. Briefly, embryos were fixed for 2 hours at room temperature. The embryos were then washed two times in PBT with Tween and permeabilized by treatment acetone at -20 °C for 7 minutes. The embryos were rehydrated and then treated with a blocking solution (2% goat serum, 1% bovine serum albumin (BSA) in PBT) for 30 minutes at room temperature. Next, the embryos were soaked in blocking solution containing the primary antibody overnight at 4°C. The embryos were then washed 5 times in PBT with 1% BSA. The embryos were subsequently exposed to the appropriate fluorescent conjugated secondary antibodies for detection. The embryos were analyzed using an epifluorescence microscope. This method uses a particular means of detection; alternative secondary reagents and visualization (or detection) methods, including, *e.g.*, chromogenic, radiographic or other methods, may be used.

A. methods

1) Maintenance of Embryos and Administration of Compounds

[0155] Fertilized zebrafish embryos were obtained by natural spawning in our aquaculture facility. To reduce variations between batches, randomized samples of embryos from 3 or 4 independent matings were used. The test medium was prepared by combining 5g of Instant Ocean Salt with 3g of $CaSO_4$ in 25 liters of distilled water, according to Westerfield, *supra*. The embryos were maintained in the test medium throughout the experiments. Embryos at 24 hours of development (with chorion) were exposed continuously for five days to chemical compounds at different concentrations of the chemical compounds and controls. In general, the concentrations ranged from 100 nanomolar (nM) to 100 micromolar (μM). Tests were repeated four times for each series of dilutions, and a standard deviation was calculated for each treatment (*see* "Statistical Methods" section). Ten embryos per concentration were exposed in a total volume of 1 ml (constant ratio of 100 μl/embryo) using a 24 multi-well plate. (Other sizes of multi-well plates, such as 96-well plates can also be used to facilitate screening.) The compounds were renewed daily. In all cases, 0.1% of dimethyl sulfoxide (DMSO) was used as a carrier solvent during the treatment. Controls with and without 0.1% DMSO were

performed in all experiments. This approach has long been used to introduce anesthetics and other chemicals into fish embryos (Westerfield, *supra*).

[0156] Experiments were carried out at a constant temperature (28-28.5°C) in the dark to protect the compounds from decomposition due to light exposure. Dead embryos were removed daily, counted, and used to calculate the Median Lethal Concentration (LC50, see "Statistical Methods" section herein). Each day, surviving embryos were analyzed visually under a dissecting binocular microscope (Zeiss, amplification 30-50X). Macroscopic malformations (such as axial defects, embryolethality, growth inhibition, general malformations, including microcephaly, macrocephaly, tail truncation, tail malformation, loss of axial structures, such as somites, *etc.*) were observed, classified, and counted to assess whole animal toxicity. Compounds that were lethal or induced these or any noted malformations or disruptions during development (*e.g.*, during the first 5 days of development) were further examined for toxic effects on organs. The embryonic developmental stage that is affected by the toxic compound can be determined. Organ toxicity can be assessed in surviving embryos using *in situ* hybridization, enzymatic assays, and immunochemistry procedures.

[0157] For therapeutic drugs screened for toxic effects, the Median Effective Concentration (EC50)(the median concentration needed to caused a desirable effect on a target) can be determined. The Therapeutic Window (TW)(*e.g.*, LC50/EC50) can also be determined; compounds exhibiting a high Therapeutic Window, such as 100 or 1,000, are good potential drug candidates because toxicity at the therapeutic concentration is low.

2) Statistical Methods

a) Estimation of LC50

[0158] For the concentrations tested with aspirin and dexamethasone in these studies, there was no partial lethality and the geometric mean of the parameters "no mortality" (0%) and "mortality" (100%) of the effect concentration was taken as the LC50 and binomial confidence limits were calculated according to Stephan, "Methods for Calculating LD50" in *Aquatic Toxicology and Hazard Evaluation* (F.L. Mayce and J.L. Hamelink eds.) ASTM STP 634, pp. 65-84. Amer. Soc. Testing Materials, Philadelphia, PA (1977).

b) Standard Deviation

[0159] The colorimetric liver stain method described above was used to obtain qualitative data (*i.e.*, changes in the size, presence, or location of the organ) and to study the significance of the variations found in each treatment. For each condition, four repetitions were performed and the statistical value, with its standard deviation, was used to prepare graphics using Microsoft Excel 97 or similar known, standard software/graphics programs.

B. Transgenic Teleosts

[0160] Adequate regulatory regions upstream from the target genes with predictive toxicity value response can be used to construct transgenic teleosts (*e.g.*, zebrafish) carrying reporter genes. The 5' upstream region of these genes is analyzed in order to use the regulatory region to control the expression of reporter genes in transgenic zebrafish. In this approach, genes isolated using subtractive library techniques are used to analyze the 5' regulatory region. To construct plasmids carrying a reporter gene, such as the Green Fluorescence Protein (GFP) under the control of those regulatory regions, those upstream regulatory regions that are adequate in size (1 or 2 kilobases) and expression profile are employed. These plasmids are used to produce transgenic fish as described in Long *et al., Development* 124:4105-4111 (1997). For example, because zebrafish are transparent, cells in transgenic zebrafish that express GFP (a reporter gene in specific organs and tissues) can be detected *in vivo* using standard fluorescence-based detection techniques; specifically, when cells expressing GFP are illuminated with blue or ultraviolet (UV) light, a bright green fluorescence is observed. Light-stimulated GFP fluorescence technique does not require co-factors, substrates or additional gene products and therefore screenings can be performed *in vivo,* and using the same embryos, toxicity effects can be monitored over time using, *e.g.*, a fluorescence plate reader. Using this screening method, many genes involved in a drug response which would otherwise be difficult to assay can be easily assessed.

C. Screening Automation

[0161] The multi-parametric methodology described above can be automated using standard instrumentation and computer software programs, permitting the screen of hundreds of compounds per week. Screening of teleost embryos can be performed in 96 multi-well culture plates containing fertilized embryos. As for other screening methods discussed above, because teleost embryos normally develop in 100 µl of water compounds and dyes can easily be added to the

medium. Furthermore, because transparent teleost embryos become opaque when they die, embryolethality is comparatively straightforward using a standard microtiter plate reader to calculate the LC50. For organ toxicity, because the cDNA microarray assays are quantitative (color fluorescence) with good confidence limits, and the GFP transgenic zebrafish can be monitored over time, the multi-well plate reader format (*e.g.*, for 96 wells or any other number of multiple wells) can be used.

[0162] The above examples are provided to illustrate the invention, but not to limit its scope; other variants of the invention will be readily apparent to those of ordinary skill in the and are encompassed by the claims of the invention.

Claims

1. A method of screening an agent for an angiogenesis activity, said method comprising administering the agent to a teleost and detecting a response in the teleost indicating angiogenesis activity.
2. The method of claim 1, wherein angiogenesis activity is decreased.
3. The method of claim 1, wherein angiogenesis activity is increased.
4. The method of claim 2, wherein the response is a decrease in normal blood vessel formation.
5. The method of claim 3, wherein the response is an increase in normal blood vessel formation.
6. The method of claim 2, wherein the response is loss of existing blood vessels.
7. The method of claim 1, wherein the teleost is an embryo, larva, or adult.
8. The method of claim 1, wherein the teleost is a zebrafish, medaka, Giant rerio, or puffer fish.
9. The method of claim 8, wherein the teleost is a zebrafish embryo.
10. The method of claim 1, wherein the teleost is a wildtype strain.
11. The method of claim 1, wherein the teleost contains a mutation in a selected gene.
12. The method of claim 1, wherein the teleost is transgenic.
13. The method of claim 1, wherein the agent is administered to the teleost by dissolving the agent in media containing the teleost.
14. The method of claim 1, wherein the agent is administered to the teleost by injecting the agent into the teleost.
15. The method of claim 1, wherein the agent is administered to the teleost in conjunction with a carrier.
16. The method of claim 15, wherein the carrier is a solvent, lipid, or peptide.
17. The method of claim 1, wherein the agent is a compound and a library of compounds is screened for angiogenesis activity.
18. The method of claim 1, wherein the agent is a nucleic acid, peptide, protein, glycoprotein, carbohydrate, lipid, or glycolipid.
19. The method of claim 18, wherein the nucleic acid is DNA or RNA.
20. The method of claim 5, wherein blood vessels are visualized by light microscopy after alkaline phosphatase staining of the teleost.
21. The method of claim 1, wherein the teleost is bleached after staining with alkaline phosphatase.

22. The method of any one of claims 1 to 21, wherein the teleost is contained in a microtiter well.

23. The method of claim 22, wherein the response is detected using a microplate reader.

Patentansprüche

1. Verfahren zum Screenen eines Wirkstoffs auf angiogene Aktivität, wobei das Verfahren die Verabreichung des Wirkstoffs an einen Teleosten und die Detektion einer Reaktion im Teleosten, die auf angiogene Aktivität hinweist, umfasst.

2. Verfahren nach Anspruch 1, worin die angiogene Aktivität verringert wird.

3. Verfahren nach Anspruch 1, worin die angiogene Aktivität erhöht wird.

4. Verfahren nach Anspruch 2, worin die Reaktion in der Verringerung normaler Blutgefäßbildung besteht.

5. Verfahren nach Anspruch 3, worin die Reaktion in der Erhöhung normaler Blutgefäßbildung besteht.

6. Verfahren nach Anspruch 2, worin die Reaktion im Verlust von existierenden Blutgefäßen besteht.

7. Verfahren nach Anspruch 1, worin der Teleost ein Embryo, eine Larve oder ein erwachsenes Tier ist.

8. Verfahren nach Anspruch 1, worin der Teleost ein Zebrafisch, Medaka, Riesenrerio oder Kugelfisch ist.

9. Verfahren nach Anspruch 8, worin der Teleost ein Zebrafischembryo ist.

10. Verfahren nach Anspruch 1, worin der Teleost ein Stamm des Wildtyps ist.

11. Verfahren nach Anspruch 1, worin der Teleost eine Mutation in einem ausgewählten Gen enthält.

12. Verfahren nach Anspruch 1, worin der Teleost transgen ist.

13. Verfahren nach Anspruch 1, worin der Wirkstoff dem Teleosten verabreicht wird, indem der Wirkstoff in einem den Teleosten enthaltenen Medium gelöst wird.

14. Verfahren nach Anspruch 1, worin der Wirkstoff dem Teleosten verabreicht wird, indem der Wirkstoff in den Teleosten injiziert wird.

15. Verfahren nach Anspruch 1, worin der Wirkstoff dem Teleosten zusammen mit einem Träger verabreicht wird.

16. Verfahren nach Anspruch 15, worin der Träger ein Lösungsmittel, Lipid oder Peptid ist.

17. Verfahren nach Anspruch 1, worin der Wirkstoff eine Verbindung ist und eine Bibliothek von Verbindungen auf angiogene Aktivität gescreent wird.

18. Verfahren nach Anspruch 1, worin der Wirkstoff eine Nucleinsäure, ein Peptid, Protein, Glykoprotein, Kohlenhydrat, Lipid oder Glykolipid ist.

19. Verfahren nach Anspruch 18, worin die Nucleinsäure DNA oder RNA ist.

20. Verfahren nach Anspruch 5, worin Blutgefäße durch Lichtmikroskopie sichtbar gemacht werden, nachdem der Teleost mit alkalischer Phosphatase gefärbt wurde.

21. Verfahren nach Anspruch 1, worin der Teleost nach dem Färben mit alkalischer Phosphatase entfärbt wird.

22. Verfahren nach einem der Ansprüche 1 bis 21, worin der Teleost in einem Mikrotiter-Well enthalten ist.

23. Verfahren nach Anspruch 22, worin die Reaktion unter Verwendung eines Mikroplattenlesegeräts detektiert wird.

Revendications

1. Un procédé de criblage destiné à déterminer l'activité d'angiogénèse d'un agent, ledit procédé comprenant les étapes consistant à administrer l'agent à un poisson téléostéen et à détecter une réponse dans le poisson téléostéen indiquant une activité d'angiogénèse.

2. Le procédé de la revendication 1, dans lequel l'activité d'angiogénèse est diminuée.

3. Le procédé selon la revendication 1, dans lequel l'activité d'andiogénèse est augmentée.

4. Le procédé selon la revendication 2, dans lequel la réponse est une diminution dans la formation normale des vaisseaux sanguins.

5. Le procédé de la revendication 3, dans lequel la réponse est une augmentation dans la formation normale des vaisseaux sanguins.

6. Le procédé de la revendication 2, dans lequel la réponse est une déperdition de vaisseaux sanguins existants.

7. Le procédé selon la revendication 1, dans lequel le poisson téléostéen est un embryon, une larve, ou un adulte.

8. Le procédé selon la revendication 1 dans lequel le poisson téléostéen est un poisson zèbre, un médaka, un réro géant ou un poisson souffleur.

9. Le procédé selon la revendication 8, dans lequel le poisson téléostéen est un embryon de poisson zèbre.

10. Le procédé selon la revendication 1, dans lequel le poisson téléostéen est une souche à phénotype sauvage

11. Le procédé selon la revendication 1, dans lequel le poisson téléostéen contient une mutation dans une gêne sélectionné.

12. Le procédé de la revendication 1, dans lequel le poisson téléostéen est transgénique.

13. Le procédé de la revendication 1, dans lequel l'agent est administré au poisson téléostéen par dissolution de l'agent dans des milieux contenant le poisson téléostéen.

14. Le procédé de la revendication 1, dans lequel l'agent est administré au poisson téléostéen par injection de l'agent dans le poisson téléostéen.

15. Le procédé de la revendication 1, dans lequel l'agent est administré au poisson téléostéen en liaison avec un porteur.

16. Le procédé de la revendication 15, dans lequel le porteur est un solvant, un lipide ou un peptide.

17. Le procédé de la revendication 1, dans lequel l'agent est un composé, et où il est effectué un criblage destiné à déterminer l'activité d'angiogénèse d'une bibliothèque de composés.

18. Le procédé de la revendication 1, dans lequel l'agent est un acide nucléique, un peptide, une protéine, une glycoprotéine, un hydrate de carbone, un lipide ou un glycolipide.

19. Le procédé de la revendication 18, dans lequel l'acide nucléique est un ADN ou un ARN.

20. Le procédé de la revendication 5, dans lequel les vaisseaux sanguins sont visualisés par microscopie optique après coloration du poisson téléostéen avec une phosphatase alcaline.

21. Le procédé selon la revendication 1, dans lequel le poisson téléostéen est décoloré après coloration avec une

phosphatase alcaline.

22. Le procédé de l'une quelconque des revendications 1 à 21, dans lequel le poisson téléostéen est contenu dans une cupule de microtitrage.

23. Le procédé de la revendication 22, dans lequel la réponse est détectée en utilisant un lecteur de microplaque.

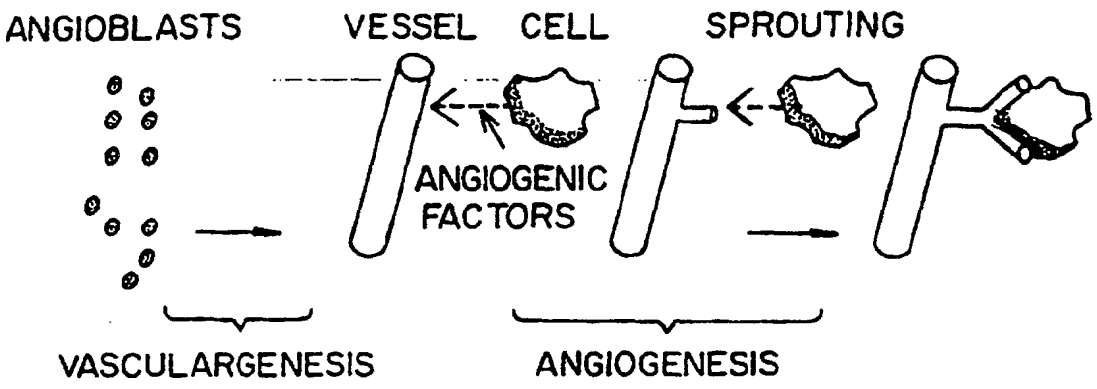
ANGIOBLASTS
VESSEL
CELL
SPROUTING
ANGIOGENIC FACTORS
VASCULARGENESIS
ANGIOGENESIS

FIG. 1.

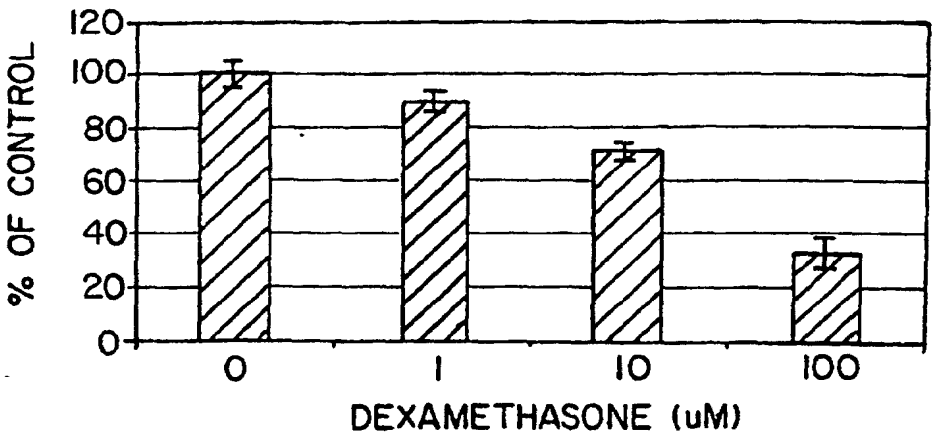
% OF CONTROL
120
100
80
60
40
20
0
0
1
10
100
DEXAMETHASONE (uM)

FIG. 13.

FIG. 2A.

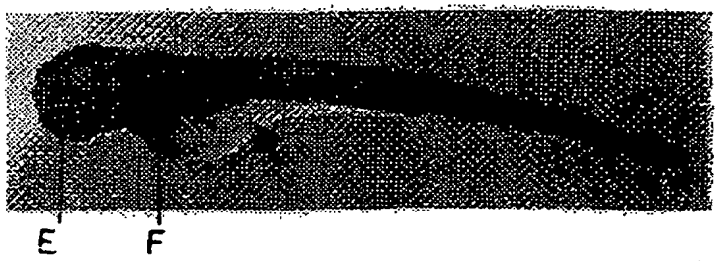

FIG. 2B.

FIG. 2C.

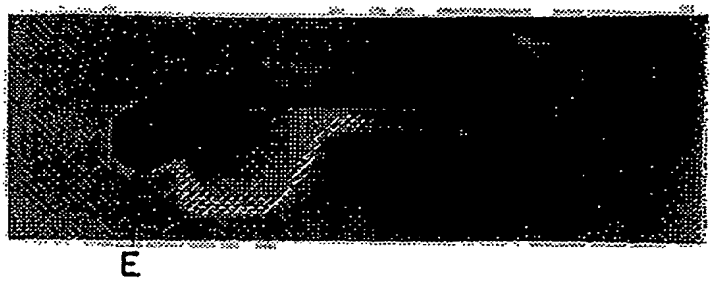

FIG. 3A.

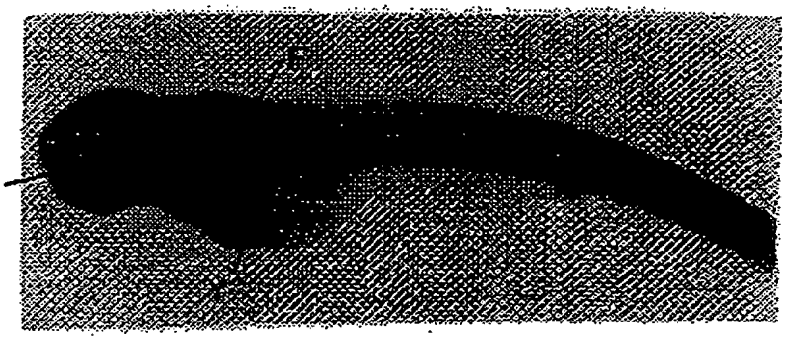

FIG. 3B.

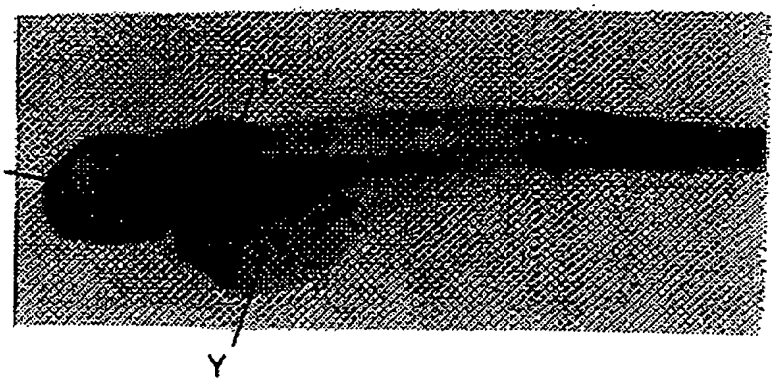

FIG. 4.

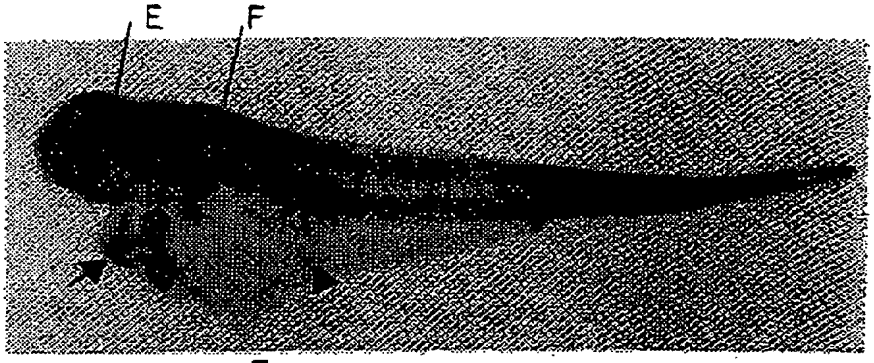

FIG. 5.

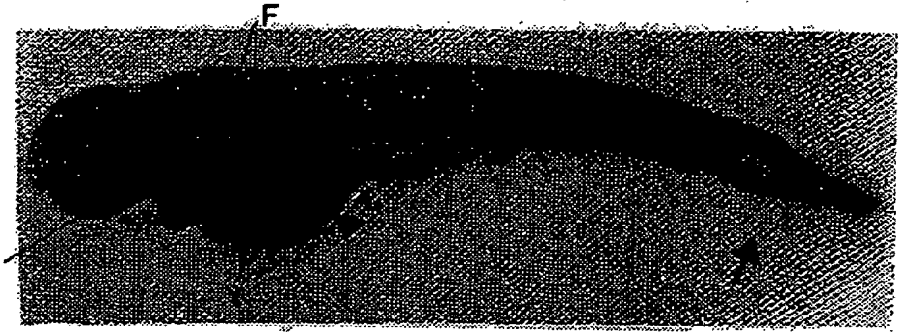

FIG. 6A.

FIG. 6B.

FIG. 6C.

FIG. 6D.

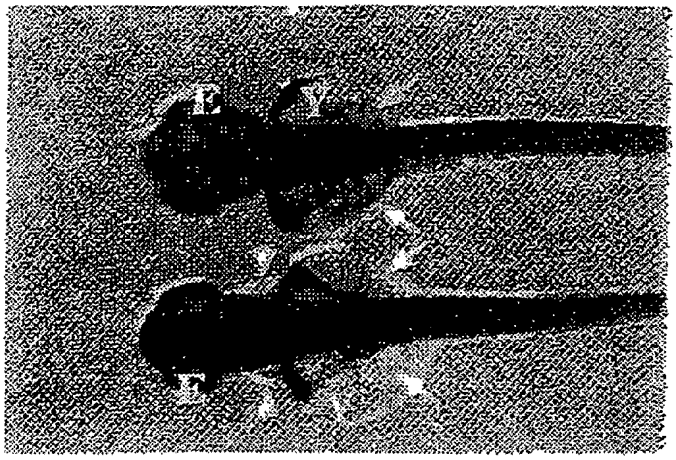

FIG. 7.

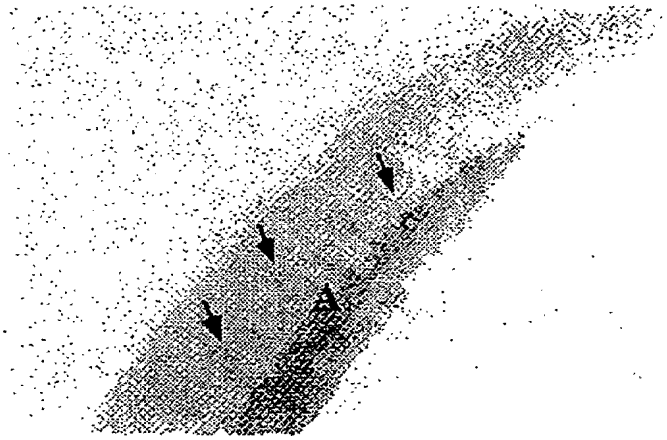

FIG. 8.

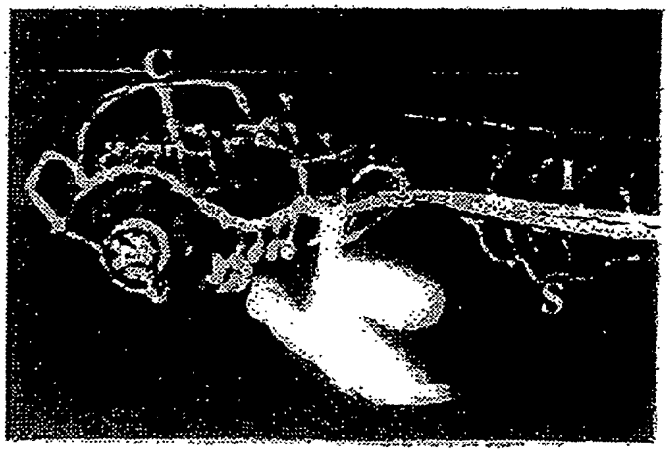

FIG. 9.